# Studies on the Genetic Epidemiology of Heritable Breast Cancer

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## Declaration

In accordance with the regulations of the University of Edinburgh, I declare that this thesis has been composed by me. The work reported in this thesis is my own with all contributions from other workers clearly indicated in the text.

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# INTRODUCTION

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# Introduction

## 1. The Cost of Breast Cancer.

World-wide, breast cancer is the most common malignant tumour affecting women. It accounted for over half a million histologically confirmed cases in 1980 which is more than 18% of all cases of malignancy (Tomatis, 1990). The contribution of this disease to the total burden of malignancy is particularly marked in the Western world in which it forms the single largest diagnostic entry in cancer registers. In developing nations as a whole, breast cancer ranks second behind cervical cancer in actual numbers of diagnoses (Parkin *et al.*, 1988).

In Scotland there were more than 2500 new cases of breast cancer in 1987; the last year for which complete records are available. 12% of this total involved women under age 45 and 0.5% women under 30 (Cancer Registration Statistics Scotland, 1990). It accounted for more than 28% of all female cancer diagnoses, a significantly higher percentage than in England and Wales. Age-standardised incidence rates are also more than 10% higher in Scotland than in the rest of Great Britain (Whelen *et al.*, 1990).

In terms of mortality, breast cancer is thankfully a more survivable disease than many other types of malignancy. Even so, in 1990 it caused 1250 female deaths in Scotland, of which nearly 7% were

under age 45 and 0.4% under age 30. Almost 4% of all deaths were due to this disease (Registrar General for Scotland Annual Report, 1990).

Over the last half century mortality rates from malignancy as a whole have risen nearly two fold. Breast cancer has more than matched this increase (Registrar General for Scotland Annual Report, 1990). The rate of increase has not slackened in the decade up to 1987 in which age-specific rates of breast cancer diagnoses rose by more than 10% in Scotland (Cancer Registration Statistics Scotland, 1990). The present life-time risk of breast cancer can be estimated at between one in twelve or thirteen for the female population. By almost every measure this disease accounts for a major proportion of morbidity and mortality in Scotland.

## 2. Risk Factors in Breast Cancer.

Women who are exposed to factors which predispose to breast cancer will be at higher risk of the disease than those who are not. Thus although one in twelve or thirteen women develop breast cancer in the population at large, many factors will increase or decrease the crude risk in population sub-groups. Some of these protective and predisposing factors are described below.

Most obviously, breast cancer incidence increases with age and this is most marked in peri-menopausal years. Geographical location is also

important as shown by European migrants who gradually adopt rates of breast cancer equivalent to the American population after emigration to the United States (Staszewski and Haenszel, 1965). By contrast, Japanese migrants are noted to maintain their traditional culture for longer and their descendants develop a westernised rate of breast cancer incidence only after one or two generations have passed (Buell, 1973). The importance of diet is controversial. Although some studies show that high saturated fat, protein and calorific intake correlate positively with breast malignancy (Lubin *et al.*, 1986; Toniolo *et al.*, 1989) and large body mass also increases risk (De Waard and Baanders-van Halewijn, 1974), other reports reveal no association between diet and breast cancer incidence (Kolonel *et al.*, 1983; Willett *et al.*, 1987). Research on Seventh Day Adventists who have a much lower saturated fat, protein and calorific intake than the general population suggest that their rates of breast cancer are roughly equivalent to population figures (Phillips *et al.*, 1980). Hormonal influences affect overall risk of breast cancer so that late menarche and early menopause are protective factors against breast cancer (Trichopoulos and MacMahon, 1972; Staszewski, 1971; Henderson *et al.*, 1988). An artificial early menopause by means of oophorectomy is similarly protective (Feinleib, 1968). The effect of pregnancy appears to be complex. Any pregnancy at advanced age increases risk of breast cancer, particularly a birth after age 35 (Brinton *et al.*, 1983; Tomatis, 1990). High parity in general protects against breast malignancy (Tulinius *et al.*, 1978; Brinton *et al.*, 1983). Lactation after childbirth may have a protective effect during pre-menopausal years and this is evident even after controlling for age at first birth (Byres *et al.*, 1985;

McTiernan and Thomas, 1986). The influence of exogenous oestrogens on breast cancer risk is still unclear but certainly appears to be small (Brinton *et al.*, 1979; Vessey *et al.*, 1979, 1982; Pike *et al.*, 1981). Oestrogen replacement therapy seems to have little adverse effect if used for a short period but may increase risk of breast cancer if prescribed for more than five years (Henderson *et al.*, 1988). Biopsy-confirmed benign breast disease may have a positive association with breast cancer (Hutchinson *et al.*, 1980) although this has been disputed (Brinton *et al.*, 1979; Chetty *et al.*, 1980). A previous diagnosis of breast cancer or previous mastectomy however significantly increases the likelihood of a second breast tumour (De Waard and Baanders-van Halewijn, 1974; Storm and Jensen, 1986). Although it is controversial as to whether therapeutic irradiation of a first breast cancer may slightly increase the risk of a second (Storm and Jensen, 1986), significant exposure to ionising radiation has been reported to increase risk of this malignancy in a dose-dependant manner (Land *et al.*, 1980). Without doubt, one of the strongest predictive factors for breast cancer is a positive family history, a recognised association for over a century (Butlin, 1887: Little, 1923; Lane-Clayton, 1926; Anderson, 1974).

Many of the above studies assign a level of risk to sub-groups which have been subjected either to predisposing or to protective factors. Comparison of risk in these sub-groups can be described through the use of risk ratios, known as relative risk. Thus a four-fold reduction in risk is found in women who have bilateral oophorectomy before age 40 compared with the general population (relative risk equals 0.25)

(Feinleib, 1968). Menopause prior to age 45 or menarche at age 16 confers half the risk of breast cancer compared to menopause after age 45 or menarche at age 11 (Trichopoulos and MacMahon, 1972; Henderson *et al.*, 1988). Childless women have three times the risk of breast cancer compared with those who have five or more offspring (Tulinius *et al.*, 1978). The same three-fold elevation in risk is found in those who have already had breast cancer diagnosed on a previous occasion (Storm and Jensen, 1986). A five-fold increase in risk is attributed to women who have their first child after 30 years of age compared with those giving birth before age 18 (Brinton *et al.*, 1983). As an example of a very small sub-group subjected to relative risk analysis; young women for whom both mother and sister have had confirmed breast cancer have a fifty-fold increase in population risk of breast malignancy between ages of 20 and 29 (Anderson, 1974).

Relative risks can tell us something about the contribution which independent factors make towards the total burden of breast cancer. They are useful in prevention of disease through identification of factors which might profitably be avoided with consequent reduction in morbidity from breast cancer. They also indicate the degree to which high-risk individuals might benefit from intensive breast screening. An accurate assessment of the value of preventative measures depends on the evaluation of factors which allow early detection of malignancy as well as risk factors for the disease itself. Population mammographic screening to detect early breast cancer has been shown to reduce mortality from breast cancer to the extent that for women over fifty years of age the absolute risk of death from this

disease is 20 - 40% less than in the non-screened group. The cost of biennial mammography within this population has been favourably compared to that of cervical screening and transplant services in terms of number of life-years saved (Miller *et al.*, 1990).

Absolute risks probably provide more directly useful information for patients than relative risks. Thus although the relative risk of breast cancer is 12:1 between ages 20 - 39 in women with a first degree relative previously diagnosed with breast malignancy compared with those who have no such familial history, the absolute risk in that age-interval is only 10% (an average of 0.5% per annum). This group however has a relative risk of 6 : 1 between ages 40 - 59. Although the relative risk compared with the general population during this later age-interval is lower than during the earlier age-interval, the absolute risk of breast cancer is greater at 14% (an average of 0.7% per annum) (Anderson, 1974).

The development of our understanding of the importance of family history in breast cancer begins with Roman historians (Sneider *et al.*, 1986). Its subsequent progress is the subject of the next section.

## 3. Confirmation of a Familial Predisposition : Historical Perspective.

The earliest known report of a familial aggregate of breast cancer appeared in a 1837 Massachusetts surgical publication. It was reported

that the affected person had "scirro-cancer" and that "frequently some of the relatives of the affected person are cancerous" (Warren, 1837). Then in 1866 the eminent physician Paul Broca published the detailed pedigree of "Madame Z". In this family multiple cases of breast and "liver" cancer extended through four generations and occurred at a younger age than expected (Broca, 1866). In London, James Paget was certain from his observations that a tendency to cancer was heritable. He realised however that such a common disease could occur as a familial cluster solely by chance (Paget, 1857). Yet in 1887, Butlin noted that affected relatives of breast cancer patients were almost invariably in one line of descent (Butlin, 1887). This confirmed that a heritable factor was the most reasonable cause for familial predisposition.

The very earliest large analysis of malignancy in relatives of cancer patients reported that observed risk was less than that expected in the general population. There were few controls however and pedigree documentation was poor by modern standards (Pearson, 1904). By contrast, two later studies in the 1920's included detailed pedigree ascertainment and revealed an increased incidence of malignancy in siblings and progeny of individuals with cancer compared with controls. Furthermore, sisters of patients for whom breast cancer was the specified malignancy had twice the population risk of breast cancer themselves (Little, 1923; Lane-Clayton, 1926). Several other reports at this time suggested a genetic susceptibility to malignancy in animals and man (Hauser and Weller, 1913; Slye, 1922; Cockayne, 1927). The possibility that early age of onset of breast cancer might be an

important pointer to genetic predisposition was reported as early as 1941 (Crabtree, 1941) and appeared to be confirmed in later studies (Jacobsen, 1946; Anderson, 1972; Lynch *et al.*, 1976, 1979). Epidemiological research revealed that first degree relatives of breast cancer patients were at particularly high risk if the proband had disease at a young age (Anderson 1974, 1977; Lynch *et al.*, 1976,1979).

Analyses which assess the risk of breast cancer in women with a strong family history can be further refined through epidemiological studies which are subjected to segregation analysis. This type of analysis not only suggests the probable mode of inheritance but can also be modified to predict the likelihood that an individual will develop breast cancer on the basis of her known family history. The principles involved in this approach are explained in the next section.

## 4. Confirmation of a Heritable Predisposition : - Segregation Analysis

In the rare heritable malignant syndromes such as retinoblastoma and familial adenomatous polyposis (FAP), inspection of a few affected families generally reveals the mode of genetic inheritance. However the genetic component involved in common malignancies such as breast cancer is less clear and its elucidation is complicated by two factors. First, a common malignancy may occur by chance within a pedigree. This confuses the apparent pattern of inheritance when it

occurs within a non gene-carrying branch of the family. Second, a gene-carrier may not develop the disease because of a relatively low age-specific penetrance of the disease-associated gene. Thus, for example, a gene-carrying mother may have offspring with heritable breast cancer but not develop the disease herself. The distribution of breast cancer in pedigrees therefore rarely conforms to simple Mendelian principles. A more complex statistical analysis is required to determine mode of transmission and is known as segregation analysis.

This kind of study involves the collection of a large series of breast cancer probands and in addition confirms disease incidence in close relatives. The relative likelihood of obtaining the observed distribution of affected and unaffected pedigree members is computed under different genetic models such as single gene autosomal dominant, recessive or polygenic inheritance. A maximal likelihood score is obtained for each hypothetical model. The score for one model may be sufficient statistically to exclude other possibilities.

The development of a unified mixed model (Morton and MacLean, 1974) has been applied to data in this kind of analysis. The model depends on accurate estimation of curves which define age-specific liability for developing disease (Falconer, 1965, 1967) to which a major predisposition locus or loci of varying hypothetical transmission type and penetrance contribute (Elston and Stewart, 1971) together with a multifactorial component and random environmental factors in an independent manner. Modifications allow for additional relatives to

be added to the pedigrees under analysis (Elston and Sobel, 1979; Lalouel and Morton, 1981).

Many segregation analysis studies have been performed on relatives of breast cancer probands in recent years. These predominantly confirm that a single gene transmitted in an autosomal dominant fashion is the model which best fits the observed data (Williams and Anderson, 1984; Andrieu *et al.*, 1988; Newman *et al.*, 1988; Bishop *et al.*, 1988; Claus *et al.*, 1991; Iselius *et al.*, 1991). Estimates of lifetime penetrance of the disease gene are remarkably consistent at 80 - 90% although predicted population gene frequency varies more widely. Studies in which pedigrees were ascertained through high-risk probands give frequencies which vary most and are considered less reliable than those based on population samples. The latters' estimates of gene frequency range from 0.0006 to 0.003 (Iselius *et al.*, 1991). This suggests that between 1 and 6 out of every 1000 women will be at very high risk of developing breast cancer as a result of an inherited predisposition. Depending on the actual population gene frequency, this would account for between 1 and 6% of all female breast cancer. The different age-incidence curves for gene-carriers and non-gene carriers however means that this proportion rises to between 4 and 20% of women diagnosed under the age of 45 (Porter *et al.*, 1993).

Following segregation analysis, further refinement of risk assessment for individuals depends on whether a gene predisposing to breast cancer is in fact responsible for disease within a particular family and whether the individual in question carries the gene. There are obvious

benefits in defining a very small high-risk group of gene-carriers through genetic screening, rather than a larger medium-risk population as presently defined through segregation analysis studies. Pharmacological or surgical prophylaxis and diagnostic organ screening will become more cost effective in this well defined sub-group. Accurate definition of the high-risk group however requires prior localisation of the susceptibility gene. The means by which this may be done through the process of linkage analysis is the subject of the next section.

## 5. Confirmation of a Heritable Predisposition in Families and Individuals :- Linkage Analysis

The approach most commonly used to locate a susceptibility gene for familial disease is known as linkage analysis (Yates and Connor, 1986). This relies on the known existence of a large number of polymorphic genetic markers scattered throughout the genome. These are called markers because they "mark" or "tag" a particular chromosomal location and can be identified in an individual through the use of molecular genetic techniques. The term "polymorphic" simply indicates that more than one allelic form of the marker is found in the population. Since somatic cells are diploid, individuals will have one allelic form on each of the two chromosomes on which that particular marker is located. The individual may be homozygous for the marker in which case the two alleles will be identical, or heterozygous in which case two different allelic forms of the marker

will exist. These markers are heritable. Thus each parent randomly contributes one or other allelic form to each of the two haploid gametes which, after zygotic fusion, proceed to constitute the alleles found in the offspring's genetic make-up.

Markers, like genes, are aligned in linear fashion along each of the forty-six chromosomes of the cell. In the absence of crossing over and recombination in meiosis (a purely hypothetical situation), a cancer susceptibility gene situated on the same chromosome as a marker of known allelic form will be found to associate with the same marker allele in all descendants who have inherited the susceptibility gene. Since crossing-over and recombination however does occur in meiosis, there is a chance that a susceptibility gene will fail to associate (segregate) with the same marker allele in the offspring as the parent. This chance is determined by the genetic distance between marker and susceptibility locus which in turn is a function of the physical distance (in DNA bases). Rates of meiotic crossing-over vary in different chromosomal regions and also differ between oogenesis and spermatogenesis. Therefore genetic and physical map distances are not exactly equivalent. Random association of susceptibility gene and marker alleles within cancer pedigrees suggest either that the marker under observation is located on a different chromosome to the susceptibility locus, or if on the same chromosome, distant enough for frequent recombination events to occur between the two. Such a phenomenon, where gene and marker are physically distant, ensures that recombinant gametes make up 50% of the total gamete pool. This proportion is known as the recombination fraction and is related to

genetic distance in centimorgans in non-linear fashion.. Where marker and susceptibility gene are in closer proximity a recombination fraction less than 0.5 will result. The physical distance between the two loci will also be related to the recombination fraction in a non linear fashion but when the fraction is small, each cM unit may represent of the order of one million base-pairs of DNA. Proximity of these loci results in the observed "linkage" of gene and marker allele within a cancer pedigree.

Using statistical methodology it is possible to analyse the marker allele data derived from members of a cancer pedigree. The likelihood that a given set of allele data might arise without the two loci being in proximity (recombination fraction equals 0.5) may be calculated. In addition the likelihood that the data might arise as a result of proximity of these loci (recombination fraction less than 0.5) may also be calculated. The ratio of the second to the first calculation (known as the "odds ratio") gives a value greater than one if it is more likely that the two loci are in close proximity and less than one if it is less likely. The logarithm of their value is therefore positive or negative depending on whether proximity is more or less likely. This logarithmic value is known as the "LOD" (logarithm of odds) score and is calculated at various hypothetical recombination fractions (Morton, 1955). Calculation of LOD scores without recourse to electronic computational aids is only possible for the simplest of pedigrees. A LOD score of greater than +3 (odds ratio greater than 1000:1) is generally required to assert linkage. This apparently strict criterion is due to the difference between prior odds for linkage

(before allele data is included) and posterior probability of linkage (which includes allele data). The prior odds for linkage of a susceptibility gene and any marker locus chosen at random throughout the genome are 2%. This is because linkage can only be asserted with statistical force if the locus and gene are in close proximity (recombination fraction less than 0.3), the odds of which in randomly chosen markers is 2%. By simplification of Bayes theorem (Emery, 1986), the approximate posterior odds for linkage are the prior odds (0.02) multiplied by the allele data odds (1000 : 1), which equates to 20 : 1 or 95%. This gives a false positive rate of only 5%, a generally accepted measure of statistical merit (Risch, 1992).

Modifications of two-point LOD score analysis (likelihood of linkage between a single marker and susceptibility gene) have been made to include data from two or more markers in proximity to the gene which is known as multi-point analysis (Lathrop *et al.*, 1984), and to test the likelihood that a proportion of families are not linked to the susceptibility gene which is known as heterogeneity analysis (Ott, 1985). The test for genetic heterogeneity is particularly important when families with very few affected relatives are analysed (Easton and Peto, 1990) or when the proportion of unlinked families is high (Durner and Greenberg, 1992). An acceptable model for use in linkage analysis studies of common malignancies depends upon accurate definition of the Mendelian trait as determined through segregation analysis. The correct mode of inheritance maximises the LOD score. Sporadic cases in the pedigree however will always reduce LOD scores, even if a correct mode of inheritance is assumed (Durner and

Greenberg, 1992). It is unfortunate that heritable breast cancer pedigrees are predicted to have a high level of sporadic disease contamination and furthermore exhibit genetic heterogeneity. These limitations in linkage analysis as applied to breast cancer have resulted in the necessity for considerable effort to collect large breast cancer pedigrees in which ascertainment of malignant disease is complete. The number of pedigrees for which data must be accumulated to reveal reliable linkage to a breast cancer locus will undoubtedly be greater than that required for the rarer strictly Mendelian malignant syndromes which are genetically homogenous, in which disease is seldom sporadic and penetrance is almost complete at a relatively young age. This is reflected in the recent successful linkage of loci for retinoblastoma, FAP, multiple endocrine neoplasia type I and II, and neurofibromatosis types I and II (King, 1990). In contrast, the family database necessary for veritable linkage studies in familial breast cancer is too large for small centres to accumulate adequate data independently. It is possible however that national and international collaboration may be able to circumvent those economy-of-scale limitations through pooling of family genetic information and centralised statistical analysis.

## 6. Elucidation of the Genetics of Heritable Malignancy.

Investigations into the genetics of well defined malignant syndromes which are inherited in an obvious Mendelian fashion might tell us a

great deal about the nature of the heritable component of more common and genetically complex malignancies such as breast cancer.

**a) Retinoblastoma**

In 1971, Knudson reported certain observations on the numerical distribution of distinct retinoblastoma primary tumours in individuals diagnosed with this malignancy (Knudson, 1971). He noted that in cases with bilateral disease the proportion of affected offspring closely approximated the 50% expected from a dominant mode of inheritance. Furthermore he recognised that a significant fraction of cases in which only unilateral retinoblastoma developed also had offspring with the disease. From the actual numerical distribution of either unilateral or bilateral tumours among those who either did or did not provide evidence for heritability of disease, statistical methods were employed which determined that a heritable case would on average develop three tumours. Poisson statistics explained why very occasionally a gene-carrier might fail to develop even a single tumour.

Knudson found that the proportion of sporadic to heritable cases was best explained in terms of a "two-hit" theory of carcinogenesis. By this model, a single gene would be responsible for protection against both heritable and sporadic retinoblastoma. If a single copy of this gene within the susceptible cell should be "hit", then that in itself would be insufficient for malignant transformation as the normal copy would continue to allow the cell to function in a non malignant way. However if the second copy should also be "hit" then the cell would

take on the malignant phenotype. According to this hypothesis the first mutation could be either somatic or germline while the second would always be somatic. Knudson calculated that the chance of any one cell susceptible to retinoblastoma receiving a somatic "hit" was very low at approximately $2 \times 10^{-7}$ per annum. The chance of that same cell receiving a second mutational "hit" would be the square of this value. If however the first "hit" were to be inherited and therefore present in every susceptible cell, then the overall chance of a malignant phenotype developing would be much greater. This explained why the heritable form of retinoblastoma resulted in a mean of three tumours per gene-carrier which compares with a figure of only seven per million non gene-carriers who develop even a single tumour. The observed incidence of heritable and sporadic forms of retinoblastoma was thereby related to germinal and somatic mutations rates (Knudson, 1975). A similar two-stage model was also proposed as likely to account for the observed age-incidence curves in sporadic and heritable breast cancer with hormonal influences affecting the kinetics of growth of normal breast tissue and consequently the likelihood of somatic mutation (Moolgavkar *et al.*, 1980).

The case for a two-stage carcinogenic model in retinoblastoma has been strengthened by experimental evidence. In 1963 a microscopic deletion involving one third of the long arm of a chromosome in the 13 - 15 group was detected in normal fibroblasts and blood lymphocytes of an infant who had developed the familial form of retinoblastoma (Lele *et al.*, 1963). The homologous chromosome appeared normal on cytogenetic metaphase analysis. Later work

revealed that the deletion was more precisely located to chromosome band 13q14 (Grace *et al.*, 1971; Knudson *et al.*, 1976; Salamanca-Gomez *et al.*, 1984). Further cytogenetic studies showed that deletion of this part of chromosome 13 or absence of the whole chromosome was a common feature of tumour cells from sporadic retinoblastoma (Balaban *et al.*, 1982; Gardner *et al.*, 1982; Benedict *et al.*, 1983a). This suggested that the same genetic locus might be involved in both forms of the disease. The gene encoding the human enzyme esterase D had previously been assigned to the same chromosomal location, namely 13q14 (van Heyningen *et al.*, 1975; Sparkes *et al.*, 1980). The enzyme activity of esterase D was found to be reduced by about 50% in individuals with both heritable retinoblastoma and a microscopic 13q deletion (Sparkes *et al.*, 1980). It was revealed that esterase D also exhibited polymorphic characteristics with two different human isoenzymic forms (Hopkinson *et al.*, 1973). In pedigrees with the familial form of retinoblastoma a single isoenzymic pattern was observed to segregate with individuals who were affected by the condition (Sparkes *et al.*, 1983; Connolly *et al.*, 1983). Linkage analysis revealed tight linkage of the esterase D gene to the locus responsible for retinoblastoma giving a maximal LOD score of greater than +3 at zero recombination.

The fact that 50% esterase D activity was associated with a cytogenetic deletion of one of the pair of chromosomes 13 within the germline in heritable retinoblastoma pedigrees was suggestive of the first "hit" in Knudson's hypothesis; a damaging genetic event at the germ-cell level which removed one of the copies of both the

retinoblastoma and esterase D genes. Since not immediately lethal, such a deletion became heritable. Evidence that a further event might be necessary for malignant transformation of any one somatic cell affected by a single chromosome 13 deletion was soon forthcoming. An individual with heritable retinoblastoma, 50% esterase D activity and a presumed sub-microscopic chromosome 13q14 deletion in all somatic cells exhibited complete absence of esterase D activity and an additional missing chromosome 13 in the retinoblastoma tumour cells themselves (Benedict *et al.*, 1983b). In the same year, another study suggested that homozygosity of the mutated retinoblastoma tumour suppressor gene could be achieved through non-disjunction in mitosis (Cavenee *et al.*, 1983). These data pointed to the second event in the somatic cells from which tumour originated and in which inactivation of the sole remaining normal copy of retinoblastoma and esterase D genes took place. In 1986 a gene was identified at chromosome 13q14, all or part of which was frequently deleted in retinoblastoma tumour cells. The RNA transcript of the gene was also found to be absent in this tumour type (Friend *et al.*, 1986). This further confirmed Knudson's hypothesis that the retinoblastoma gene, when mutated, is recessive at the cellular level in that both copies of the gene require inactivation for tumorigenesis. At the level of the whole organism however the predisposition to retinoblastoma is transmitted from generation to generation in an autosomal dominant fashion.

The observations that familial retinoblastoma arises from an inactivated "tumour suppressor gene" contrasts with the concept of action of dominant oncogenes (Huebner and Todaro, 1969) in which

normal "proto-oncogenes" become activated and their overactivity stimulates the tumorigenic process. Activation of oncogenes can occur through a number of pathways such as retroviral infection, chromosomal translocation, limited mutation and genetic amplification (Spandidos and Anderson, 1989).

Very recently, inheritance of a mutated copy of the RET proto-oncogene, localised to chromosome 10q11.2, has been implicated in the autosomal dominant malignant traits of Multiple Endocrine Neoplasia (MEN) types 2A and 2B, and also Multiple Thyroid Carcinoma (Mulligan *et al.*, 1993, 1994). The RET proto-oncogene encodes a tyrosine kinase receptor; these are cell surface molecules which transduce signals for cellular differentiation. The RET proto-oncogene is activated, structurally rearranged and over-expressed in tumour cells from medullary thyroid carcinoma and phaeochromocytoma. Germline mutations in the RET gene which predispose to MEN2A typically involve bases which code for conserved cysteine residues. Since only one mutant allele is sufficient for both inheritance of the trait in the germline, and additionally tumour development in susceptible individuals, it appears likely that a dominant oncogene rather than a classic tumour suppressor gene mechanism is acting in this syndrome (Mulligan *et al.*, 1993). Interestingly, inactivation of the RET proto-oncogene through mutation appears to cause the non-malignant heritable trait of Hirschsprung's Syndrome which is characterised by an aganglionic lower colonic segment, indicating the wide-ranging role which the

RET gene plays in control of growth and development (Edery *et al.*, 1994).

Although it is recognised that oncogenes do not have an important role in tumorigenesis through control of cell proliferation, in most familial malignant traits oncogenic activity is considered to be particularly crucial subsequent to the initial transformation stage (Steel, 1989). The importance of an "anti-oncogene" or "tumour suppressor gene" in the development of familial retinoblastoma has led to the search for similar genes in other heritable malignant conditions transmitted in autosomal dominant fashion, such as F.A.P.

**b) Familial Adenomatous Polyposis (FAP)**

The report of a cytogenetic germline deletion in chromosome 5q in a mentally retarded individual who exhibited features of Gardner syndrome (FAP plus extra-colonic manifestations) hinted at the possible location of a tumour suppressor gene in this condition (Herrera *et al.*, 1986). Linkage analysis studies confirmed the position of the susceptibility gene for FAP on chromosome 5q21 (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Nakamura *et al.*, 1988a; Dunlop *et al.*, 1989). The gene was identified in 1991 (Kinzler *et al.*, 1991; Nishisho *et al.*, 1991; Groden *et al.*, 1991; Joslyn *et al.*, 1991).

The distinction at the cellular level between dominant oncogenes and recessive tumour suppressor genes is on occasions difficult to maintain. For example, analyses of the adenomas which precede

colorectal carcinoma in FAP seldom yield evidence that a second allele is lost at the 5q locus, suggesting that the tumour suppressor gene is co-dominant in the premalignant lesion (Lancet Editorial, 1989).The requirement for a second event to inactivate the FAP tumour suppressor gene, as predicted in Knudson's hypothesis and confirmed in retinoblastoma, has however been confirmed in carcinomas arising in FAP.

The search for mutated tumour suppressor genes in familial malignancy has thus far yielded an increasing number of susceptibility genes. This contrasts with a relative paucity of evidence for oncogene involvement discovered to date. The best chance of elucidating the genetic component in heritable breast cancer therefore appears to be through the identification of a susceptibility locus with tumour suppressor gene characteristics (Steel *et al.*, 1991).

**c) Syndromes of Breast Cancer.**

Epidemiological studies suggest that there may be distinct heritable breast cancer syndromes which can be differentiated by the particular types of non breast malignancy which cluster within them. These may be classified into seven groups (Lynch and Kullander, 1987a);

**(i)** Site-specific heritable breast cancer

**(ii)** Breast-ovarian cancer

**(iii)** Breast cancer associated with gastro-intestinal malignancy (Lynch type II syndrome)

**(iv)** Li-Fraumeni syndrome

**(v)** Cowden's disease

**(vi)** Gorlins syndrome

**(vii)** Ataxia telangiectasia

It appears that the majority of familial aggregates of breast cancer are best classified within groups (i), (ii) and (iii). The identification of a purely site-specific pedigree is particularly difficult because a large family will inevitably have some sporadic malignancies which will affect classification (Lynch and Kullander, 1987a). It is certainly apparent that extension of pedigree data from small site-specific breast cancer families frequently result in the identification of cases of ovarian and gastro-intestinal malignancy elsewhere in the pedigree (Steel *et al.*, 1991). Diagnosis of Lynch type II (hereditary non polyposis colorectal cancer) syndrome requires at least one colon cancer diagnosis under age 50 (Hakala *et al.*, 1991; Lynch *et al.*, 1991). There are many examples of breast cancer pedigrees which fulfil some but not all of the strict criteria for this syndrome (Lynch *et al.*, 1991). Furthermore ovarian cancer is an important feature of Lynch type II families (Lynch and Kullander, 1987b).

Until recently it was therefore considered possible that groups (i), (ii) and (iii) were different phenotypic expressions of the same genotypic germline lesion. New data, however, has linked hereditary non-polyposis colorectal cancer (HNPCC) to mutations in mismatch repair genes on chromosomes 2p22-21 (Fishel *et al.*, 1993) and 3p21-23 (Bronner *et al.*, 1994). Mismatch repair genes code for enzymes which

repair mismatched nucleotides. These arise either through physical damage of genomic DNA, or through misincorporation during DNA replication, or through genetic recombination of different parental DNA sequences. Mismatch repair genes play an indispensable role in DNA replication fidelity, including stabilisation of dinucleotide repeat elements. Dinucleotide repeats are notably unstable in HNPCC. Defects in any of these genes results in a general elevation of spontaneous mutation rates.

The major features of Li-Fraumeni syndrome (Birch, 1990, 1991) include early age of diagnosis of malignancy with excess soft tissue sarcoma, bony sarcoma, adrenocortical carcinoma, acute leukaemia, central nervous system malignancy and breast cancer (Birch *et al.*, 1990). Widely accepted criteria for this syndrome are based on the definition of a proband with sarcoma under age 45, a first degree relative with cancer in this age interval and another first or second degree relative with either cancer before age 45 or sarcoma at any age (Li *et al.*, 1988). Recently the germline mutation responsible for perhaps more than 50% of this family syndrome has been pin-pointed to the p53 gene on the short arm of chromosome 17 (Malkin *et al.*, 1990; Srivastava *et al.*, 1990; Metzger *et al.*, 1991; Santibanez-Koref *et al.*, 1991a, 1991b; Iavarone *et al.*, 1992; De Fromental and Soussi, 1992).

The p53 gene encodes a 53 kiloDalton, 393 amino acid nuclear phosphoprotein. Since aberrent expression of the gene induced malignant transformation, p53 was initially classified as an oncogene.

Subsequent analysis, however, determined that only mutant p53 was able to transform cells, whereas wild-type p53 failed to do so.

p53 is mutated in a large number of human malignant types (Levine *et al.*, 1991) and much evidence now reveals p53 to be a tumour suppressor gene (Lane and Benchimol, 1990; Milner and Medcalf, 1991; Sidransky *et al.*, 1992a). For example, transgenic p53-null mice develop normally yet exhibit a high tumour incidence (Donehower *et al.*, 1992). Normal p53 is induced to accumulate in cells with radiation-damaged DNA. So crucial is wild-type p53 to genomic stability, that this accumulation occurs via a post-transcriptional mechanism, so that mutated p53 cannot be produced through transcription of new RNA from a potentially damaged template (Kastan *et al.*, 1991). p53 thereby mediates a transient inhibition of replicative DNA synthesis via arrest of the cell cycle at G1. Thus malignant clone formation is inhibited, either to allow repair of damaged DNA prior to division, or to mediate controlled cell death (apoptosis).

Wild-type p53 binds DNA in a sequence-specific fashion. Unlike wild-type p53, mutant p53 protein alone, or normal p53 protein complexed with viral or hman oncogenes is able to bind to DNA. The p53 protein core (amino acids 97 - 292) is in large part evolutionarily conserved and determines p53 conformation, specific binding to DNA sequences and also sequence-specific transcriptional activity (Greenblatt *et al.*, 1994). Structurally, this region consists of two large

loops held by a zinc atom, and additionally a loop-sheet-helix motif which interacts in the major groove of DNA (Cho *et al.*, 1994).

The majority of point mutations found in human tumour types occur in five domains which are highly conserved throughout species. This emphasises the critical nature of these conserved sequences in normal p53 function. Four of the five highly conserved regions (HCR's) are located in the central core of the gene (HCR I encompasses amino acid residues 13 - 19, HCR II; residues 117 - 142, HCR III; residues 171 - 181, HCR IV; residues 234 - 258, and HCR V; residues 270 - 286). Exons 5,7 and 8 encompass most of this central core's highly conserved regions in which p53 gene mutations are largely concentrated.

Beyond the central core of p53, the amino end of the gene controls transcriptional activity of genes downstream of the p53 binding site. In contrast, the oligomerization function of p53 resides at the carboxy terminus of the gene, outside the central region prone to frequent mutation. Oligomerization in the form of dimers and tetramers are required for DNA binding and transactivation of the protein (Greenblatt et al., 1994).

In keeping with data in sporadic tumours, the majority of mutations in Li-Fraumeni pedigrees occur within the central core of the p53 gene; frequently within highly conserved domains (De Fromental and Soussi, 1992).

Although the p53 gene is mutated in at least 13% of sporadic breast tumours (Prosser *et al.*, 1990), analyses of non Li-Fraumeni pedigrees in which breast cancer is the predominant malignancy do not commonly reveal mutations in this gene (Prosser *et al.*, 1991).

Cowden's disease is an autosomal dominant condition producing multiple hamartomata of skin and oral mucosa (Steel *et al.*, 1991). Additional organs are involved and perhaps 50% of affected women develop breast cancer (Brownstein, 1987).

Gorlins syndrome (multiple basal cell carcinoma) is similarly autosomal dominant. In this syndrome basal cell epitheliomas occur at young age in association with gynaecological and central nervous system malignancy. Risk of breast cancer is also increased (Steel *et al.*, 1991).

Ataxia telangiectasia, transmitted as an autosomal recessive trait, is associated with an increased risk of development of immunological malignancy and solid tumours in individuals homozygous for the disease gene (Hecht and Hecht, 1990). Heterozygotes such as mothers of affected individuals are also at increased risk of various tumours (Swift *et al.*, 1990; Borresen *et al.*, 1990). In these individuals the gene confers a risk of breast cancer three-fold greater than for the general population (Swift *et al.*, 1987). Recently the gene has been mapped to the long arm of chromosome 11 (Gatti *et al.*, 1988; Wei *et al.*, 1990; Ziv *et al.*, 1991).

It is possible that all these syndromes with which breast cancer is associated are caused by different genetic events in different chromosomal regions. The consequence of this would be genetic heterogeneity of heritable breast cancer. To date, no case of familial breast cancer in association with a germline microscopic chromosomal aberration has come to light (Steel *et al.*, 1991). It is evident from the genetic elucidation of heritable retinoblastoma and FAP however that continuing examination of constitutional chromosomes in affected members of breast cancer pedigrees would be worthwhile in order to localise putative susceptibility genes and avoid the laborious process of exclusion of large areas of the genome through linkage analysis.

Attempts to locate sites of candidate tumour suppressor genes in familial breast cancer have involved work on sporadic tumours and linkage analysis in affected pedigrees. These are reviewed in the final section.

## 7. Identification of a Susceptibility Locus for Heritable Breast Cancer.

### a) Cytogenetic Analysis.

Cytogenetic analysis of breast tumours is technically difficult and yields many abnormalities. Chromosomal rearrangements however do seem to occur in some regions more frequently than others and at least twenty-one sites of recurrent breakage have been identified. These are 1p32, 1p22, 1p13, 1p11/12, 1q11/12, 1q21, 1q23, 1q25, 1q32, 2q11,

2q33, 3p11/12, 5q13, 6q16, 7q11, 8p11, 11q13, 12q24, 16q21, 17p11 and 22p and constitute less than 9% of the total genome (Rodgers *et al.*, 1984; Pathak and Goodacre, 1986; Ferti-Passantonopoulou and Panani, 1987; Gerbault-Seureau *et al.*, 1987; Mitchell and Santibanez-Koref, 1990). Precise localisation is only possible at some sites, and many anomalies defy assignment to specific bands (Steel *et al.*, 1992).

**b) Loss of Constitutional Heterozygosity Studies.**

A second method by which tumour suppressor gene loci have been identified through sporadic breast tumour studies involves analysis of marker alleles in normal somatic and tumour cell DNA from a number of affected individuals. Most patients will be constitutionally heterozygous for highly informative polymorphic markers. However microscopic and sub-microscopic genetic deletion in tumour DNA is a frequent occurrence and will reveal itself through loss of allele heterozygosity (apparent homozygosity) at the marker over which the deletion extends. Background loss of allele heterozygosity is reported in 5 - 20% of tumours and in this context is without significance (Steel *et al.*, 1992). Yet when a larger proportion of tumours exhibit loss of constitutional heterozygosity at a particular locus, there is a suggestion that this site might be close to a tumour suppressor gene which requires inactivation for tumour progression (Lasko *et al.*, 1991). The possibility that localised allele loss in tumour DNA may indicate the presence of a tumour suppressor gene in close proximity has been confirmed in studies on retinoblastoma, and also on colorectal cancer in which a large proportion of tumours exhibited allele loss along the

long arm of chromosome 5, close to the susceptibility gene for FAP (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988, Sasaki *et al.*, 1989).

Loss of heterozygosity studies have also yielded information on tumour suppressor gene loci likely to be involved in early rather than late tumour development. This approach however relies upon the ability to distinguish between early and late tumour histology. In colorectal tumorigenesis, adenomas proceed through well defined stages prior to development of frank malignancy. Molecular analyses reveal that events important early in the malignant process include allele deletion at 5q and ras oncogene mutation. Later events include loss of heterozygosity at 18q and also 17p close to the p53 gene locus (Vogelstein *et al.*, 1988). Although this evidence might appear to exclude p53 as a candidate susceptibility gene for hereditary non polyposis colorectal cancer, its responsibility for a large proportion of the Li-Fraumeni cancer family syndrome (to which colorectal carcinoma does not contribute) is well known. It therefore appears that single tumour suppressor genes may be important either in the primary tumorigenic event or at some later stage in tumour progression, depending on the susceptibility of the tissue type. From a histological standpoint it is difficult to differentiate early from late cancer of the breast. Certainly in benign atypical proliferative breast disease, which is itself a risk factor for breast malignancy, loss of allele heterozygosity at any locus has yet to be reported (Skolnick *et al.*, 1990).

For breast cancer in general there are at least twelve chromosomal locations in which excessive loss of constitutional heterozygosity occurs. These involve chromosomes 1p, 1q, 3p, 6q, 7p, 11p, 13q, 16q, 17q, 18q and two regions on 17p (Lasko *et al.*, 1991; Andersen *et al.*, 1992; Steel *et al.*, 1992). Although the confirmed region of allele loss on chromosome 1p32-36 overlaps a cytogenetically defined break-point on the same chromosome arm (1p32), throughout the genome there is only limited concordance of results from cytogenetic and allele loss studies (Steel *et al*, 1992). It is suggested therefore that these two analytical approaches are of value in defining a multitude of tumour suppressor genes which have a variety of roles in the tumorigenic process, either early or late. However their ability to identify candidate susceptibility loci may be compromised by a great deal of background noise from other tumour suppressor genes. The large number of such loci means that only through a combined collaborative approach by many laboratories would wasteful repetition of work be avoided. In recent times this has been made possible through the efforts of the European breast cancer linkage consortium. This consortium of laboratories has agreed to co-ordinate their activities in the search for a heritable breast cancer gene. Linkage data on candidate loci are made available at an early stage to facilitate this process. Loci which appear to give positive evidence for linkage may then be further examined by means of joint analysis of pooled family data.

**c) Attempts at Linkage Analysis.**

As expected, some attempts at linkage analysis in breast cancer have centred on those regions in which loss of allele heterozygosity occur in a high proportion of tumours such as chromosome 1p (Genuardi *et al.*, 1989; Bieche *et al.*, 1990). A maximal LOD score of +1.88 at zero recombination distance from the polymorphic rhesus blood group was reported (Ferrell *et al.*, 1989) but this finding was later refuted (King, 1989). Reports of positive linkage at the glutamic-pyruvic transaminase locus on chromosome 8q (maximal LOD score of +1.95) (King *et al.*, 1980, 1983) and the ABO blood group locus on chromosome 9q (maximal LOD score of +1.99) (Skolnick *et al.*, 1984), have also been challenged (Cannon *et al.*, 1983; Bishop *et al.*, 1988; Ferrell *et al.*, 1989). Loss of constitutional heterozygosity at 17p13, telomeric to the p53 gene locus, has been reported in over 60% of breast tumours (MacKay *et al.*, 1988; Coles *et al.*, 1990). A LOD score of +1.8 at this locus (known as YNZ 22.1) was calculated for a single pedigree with familial breast cancer (MacKay, 1989), although other centres could not confirm linkage at this site (Hall *et al.*, 1989).

Away from the short arm of chromosome 17, an analysis of linkage in a series of American pedigrees suggested that a marker known as CMM86 mapping to the long arm of this chromosome at 17q21-22 produced a maximal LOD score of +5.98 at zero recombination in families in which breast cancer tended to occur at a young age (Hall *et al.*, 1990). LOD scores were markedly positive in six out of seven such pedigrees. In contrast, linkage was refuted in families where breast

cancer was diagnosed at an older age, with highly negative scores reported in ten out of sixteen such pedigrees. Further evidence for the existence of a gene responsible for familial breast cancer in proximity to this locus was provided in a French report in which three out of five large pedigrees with breast and ovarian cancer exhibited strong evidence in favour of linkage at this locus (Narod at al., 1991). These studies suggested that heritable breast cancer might be a heterogenous entity with a particularly high likelihood of a mutated susceptibility locus on chromosome 17q existing in families in which breast cancer at young age or ovarian cancer at any age formed part of the syndrome. The European breast cancer linkage consortium found that these data were consistent with a range of possible interpretations; at one extreme, tight linkage of the two loci in only 20% of families, and at the other, a disease gene located up to 20cM from this marker with up to 90% of families linked (Easton *et al.*, 1993). As yet there is a paucity of data from breast tumour loss of heterozygosity studies at CMM86, but a particularly high degree of allele loss has been found in sporadic ovarian cancer at CMM86 and regions just telomeric to this site (Eccles *et al.*, 1990; Russell *et al.*, 1990; Sato *et al.*, 1991; Foulkes *et al.*, 1991). Loss of heterozygosity at CMM86 is also apparent, but less markedly so, in sporadic breast tumours (Coles *et al.*, 1990; Cropp *et al.*, 1990).

A number of candidate genes are known to lie on the long arm of chromosome 17 (Solomon and Barker, 1989; Hall *et al.*, 1990; McKusick, 1990). The proteins which they encode include the trans-membrane receptor Erb B2 amplified in many primary breast tumours

(Coussens *et al.*, 1985; Di Fiore *et al.*, 1987), 17 ß-oestradiol dehydrogenase (17HSD) which converts oestrone to oestradiol (McKusick, 1990), homeobox 2 (hox 2) critical for early development (Hall *et al.*, 1990), nm23 which is expressed in association with breast cancer lymph node metastasis (Steeg *et al.*, 1988; Bevilacqua *et al.*, 1989), the human prohibitin gene which is both anti-proliferatory and mutated in some sporadic breast tumours (Sato *et al.*, 1992), and INT2 which is homologous with one of the mouse mammary tumour virus integration site (Rosengard *et al.*, 1989). All of these genes are located centromeric to CMM86 within bands 17q12-21.

To determine whether a susceptibility gene for heritable breast cancer exists at this site and to localise it more precisely, linkage studies involving markers which span the region 17q12-21 are required.

# MATERIALS AND METHODS

1. **Pedigree collection and ascertainment.**

2. **Materials and suppliers.**

3. **Buffers and solutions.**

4. **DNA extraction and purification.**
   a) Blood.
   b) Paraffin embedded tissue.
   c) Quantification of extracted DNA.

5. **Allele data for linkage analysis.**
   a) Restriction Fragment Length Polymorphisms (RFLPs).
      i) Variable number of tandem repeats (VNTRs).
      ii) DNA digestion.
      iii) Agarose gel electrophoresis.
      iv) Southern blotting.
      v) Hybridisation.
   b) Microsatellites.
      i) Primer preparation.
      ii) Microsatellite amplification.
      iii) Polyacrylamide gel electrophoresis.
      iv) DNA electroblotting and hybridization with radiolabelled (dCdA) probe.

6. **p53 mutation analysis.**
   a) Denaturant Gradient Gel Electrophoresis. (DGGE)
      i) Primer preparation.
      ii) Exon amplification
      iii) Polyacrylamide gel electrophoresis and staining.
   b) Sequencing

7. **Linkage analysis.**

8. **Structural chromosome analysis**

## Materials and Methods.

### 1. Pedigree Collection and Ascertainment.

Patients claiming a strong family history of breast cancer were referred by interested medical practitioners for inclusion in epidemiological and genetic research based at the MRC Human Genetics Unit. Pedigrees were extended as far as possible by patient interview and details of births, marriages and deaths confirmed by recourse to registration data. This involved ascertainment of all first and second degree relatives of the proband as a minimum requirement. In Edinburgh, capital city of Scotland, West Register House maintains all Scottish registrations including certificated cause of death. These are complete from 1855 and Parish records are available prior to this. Medical genealogists based at the MRC Human Genetics Unit have unique privileged access to these valuable documents to enable verification of pedigree data. Causes of death were ascertained from these Registrar General for Scotland`s records and histological diagnoses of cancer confirmed from pathological departments and other hospital sources. Details of thirty seven pedigrees were collected in this way.

Experience of sib-pair linkage analysis (in which two affected sisters act as a basic pedigree) suggested that a large number (>50) of such families would be necessary for significant linkage to be revealed. Therefore only pedigrees with three or more cases of

breast cancer in blood lineage were included in the linkage study. This amounted to a total of fifteen families whose basic pedigrees are shown in Figure 1. There was no bias towards collection of pedigrees with other particular types of cancer, although all cancer cases were recorded together with age at histological diagnosis. Neither was age of onset of breast malignancy a criterion in the selection of pedigrees for linkage analysis. Survival data was collected at the time of pedigree ascertainment for individuals who developed malignancy.

Two additional breast cancer pedigrees were investigated. The first was ascertained through a proband who was one of 136 breast cancer patients who had tumours screened for p53 mutations (Prosser *et al.*, 1992). This individual was the only one found to have a mutation in the germline. Her pedigree was discovered to contain a further two breast cancer cases though no first degree relative was affected. The second family was ascertained as part of a study into cancer risk in relatives of osteosarcoma patients (Porter *et al.*, 1992). Two close relatives were found to have breast cancer in this pedigree which also fulfilled criteria for Li-Fraumeni cancer family syndrome classification. Both pedigrees are shown in Figure 2.

**Figure 1**

Fifteen Edinburgh breast cancer pedigrees subjected to linkage analysis.

Br : unilateral breast cancer

Brx2 :bilateral breast cancer

Ov : ovarian cancer

Figure 1

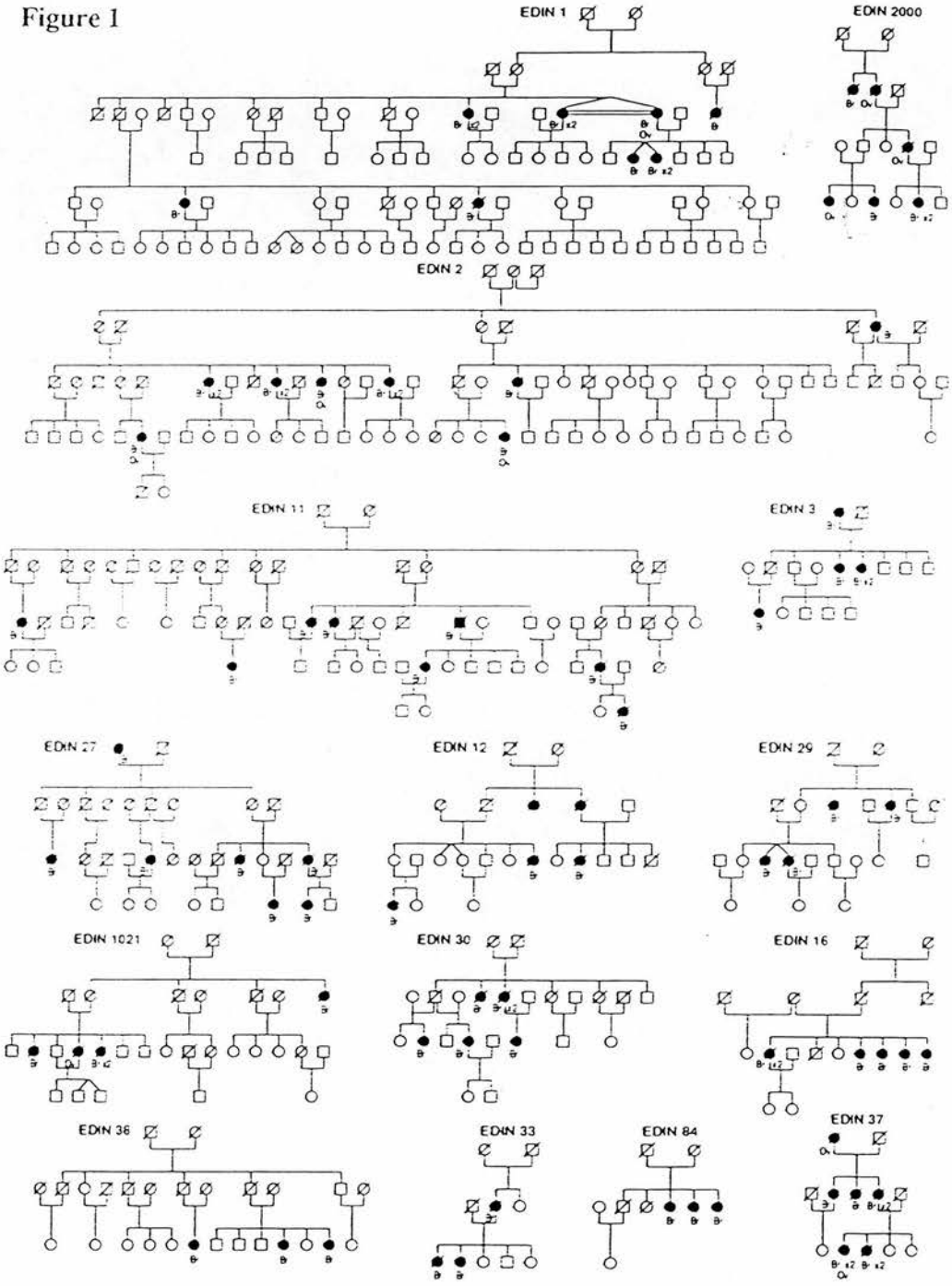
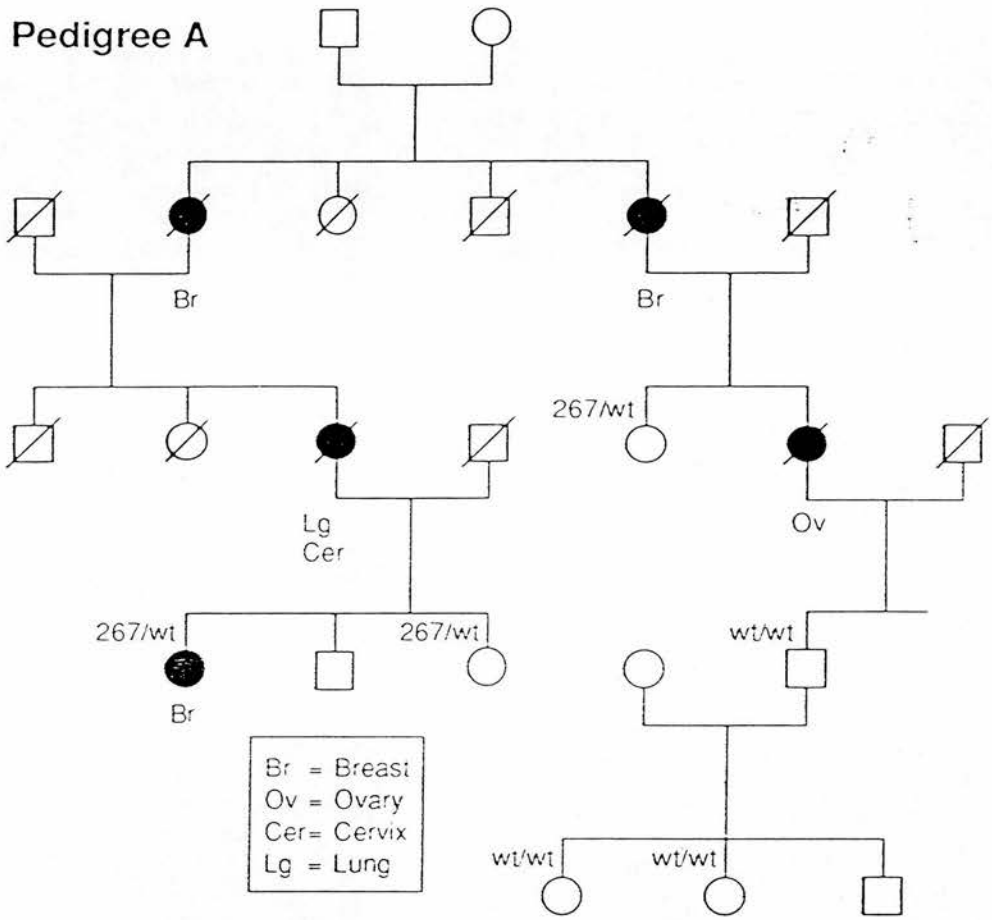

EDIN 1
EDIN 2000
EDIN 2
EDIN 11
EDIN 3
EDIN 27
EDIN 12
EDIN 29
EDIN 1021
EDIN 30
EDIN 16
EDIN 36
EDIN 33
EDIN 84
EDIN 37

**Figure 2**

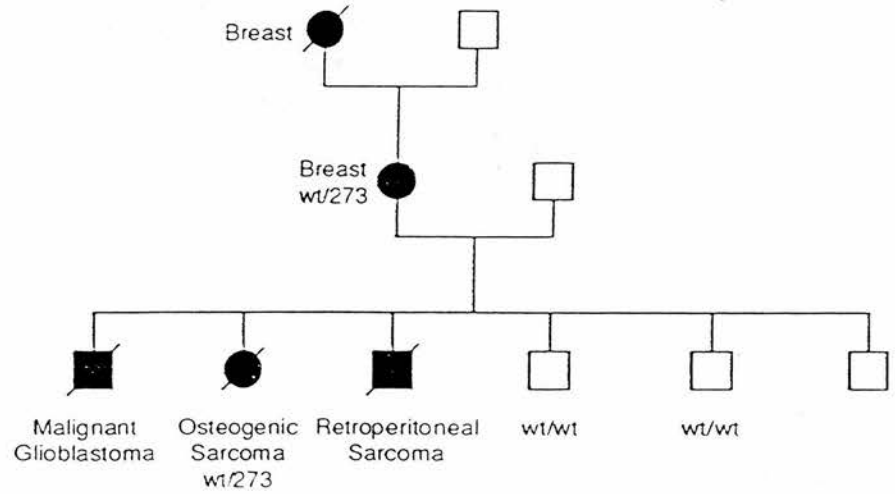
Two breast cancer pedigrees with inherited p53 germline mutations.

# Figure 2

## Pedigree A




## Pedigree B



## 2. Materials and Suppliers.

### Alpha Lab

Plastic disposable transfer pipettes/ pastettes

### Amersham

Hybond N nylon membrane

Multiple DNA labelling/random priming kit RPM 1600Y

### Applied Biosystems

DNA synthesisers 381A and 391A

### BDH-Analar

Acrylamide

Ammonium acetate

Bis-acrylamide

Chloroform

Dimethyldichlorosilane

Ethanol

Ethylene diamine tetra acetate (EDTA)

Glacial acetic acid

Hydrochloric acid

Isoamyl alcohol

Isopropyl alcohol

**BDH-Analar (continued)**

Methanol

Proteinase K

Sodium chloride

Sodium citrate

Sodium hydroxide

Sucrose

Tris borate

Tris hydrochloride

**Becton Dickinson**

Falcon tubes (50ml)

**Bethesda Research Laboratories**

Agarose

Agarose gel electrophoresis tank Model H4

Polyacrylamide gel electrophoresis tank Model S2

**Bibby**

Heating/stirring plate

**Biometra**

Automated thermocycler TRIO-thermoblock

**Boehringer Mannheim**

Nucleotides dATP, dCTP, dGTP, dTTP

Taq 1 restriction endonuclease

Taq 1 restriction enzyme buffer

Taq 1 DNA polymerase

Taq 1 DNA polymerase 10X reaction buffer

Tris base

**Cecil**

Double beam spectrophotometer CE-594

**Clingo-rap**

Clingfilm

**Crouzet**

Circulation pump 10 r.p.m. (type 82344)

**E-C Apparatus corporation**

D.C. Power source EC-105

**Fisons**

Clifface freezer

**Fotodyne**

Fotoprep I Ultraviolet transilluminator

**Fuji**

X-ray film processor RGII

**Gilson**

Disposable transfer pipettes

**Grant**

Oven/Agitator SS40-2

Waterbath SU6

**Heto**

Vacuum centrifuge Hetovac VR1

**Hybaid**

Glass tubes HB-OV-BL

Rotating Hybridization oven

**Ilford**

Fast tungstate intensifying screen x-ray cassette

**Jencons**

Vortex mixer

**Kleenex**

Absorbant paper towels

**Kodak**

X-AR Omat film

**Leitz**

Wetzler microtome

**LKB**

Power supplies 2103 and 2197

**Mettler**

PM4600 balance

**Mini Instruments Ltd**

Geiger counter

**Mitsubishi**

Video copy processor

**MSE Scientific Instruments**

Microcentaur 13000 eppendorf microcentrifuge

Soniprep 150 ultrasonic disintegrator

**Polaroid**

MP-4 land camera

**Rathburn Chemicals**

Phenol

**Scandinova**

4°C refrigerator

**Scot**

Scotfresh regular paper towels

**Scotlab**

Eppendorf tubes (1.5ml)

PCR tubes (disposable 60µl)

**Sellotape**

Autoclave tape

**Sigma**

Agarose

Ammonium hydroxide

Ammonium persulphate

Bovine serum albumin (BSA)

Bromophenol blue

Centrifuge (3MK with Nr12056 rotor)

Ethidium bromide

Ficoll

Formamide

Light mineral oil

Polyethylene glycol (PEG) 6000

Polyvinyl pyrrolidone (PVP)

RNAase-A

Salmon sperm DNA

Sodium dodecyl sulphate (SDS)

**Sigma (continued)**

Sodium iodide

Sodium pyrophosphate

N,N,N',N', Tetramethylethylenediamine (TEMED)

TWEEN 20

Urea

Xylene

Xylene cyanol

**Sorval**

Centrifuge (3MK)

**Sterilin**

Sterile universal containers

**Stratagene**

Ultraviolet transilluminator (U.V. stratalinker 1800)

**Transatlantic plastics**

Sealing polythene

**United States Biochemicals (USB)**

Sequenase DNA sequencing kit

**Whatman**

3MM Filter paper

17MM Filter paper

**Wifug**

Benchtop centrifuge (500E)

## 3. Buffers and Solutions

**Lysis buffer :** 0.1M Tris HCl, 20mM NaCl, 1mM EDTA

**Resuspension buffer :** 10mM Tris HCl, 150mM NaCl, 10mM EDTA

**T.E. buffer :** 10mM Tris HCL (pH7.5), 0.5mM EDTA

**Digestion buffer :** 50mM Tris base (pH8.5), 1mM EDTA, 0.5% TWEEN 20

**Running buffer :** 100mM EDTA, 0.25% Bromophenol blue, 30% sucrose

**20X TAE buffer :** 484g Tris base, 114.2ml acetic acid, 200ml 0.5M EDTA in 5l

**Denaturing solution :** 5g NaOH (0.5M), 219.15g NaCl (1.5M) in 2.5l

| | |
|---|---|
| **Neutralising solution :** | 292.9g NaCl (2M), 394g Tris base (1M) in 2.5l, add HCl to pH 5.5 |
| **20X SSC buffer :** | 876g NaCl, 441.2g Sodium citrate in 5l |
| **0.1X SSC wash : (high stringency)** | 50ml 10% sodium pyrophosphate, 25ml 20% SDS, 25ml 20X SSC in 5l |
| **0.5X SSC wash :** | 50ml 10% sodium pyrophosphate, 25ml 20% SDS, 125ml 20X SSC in 5l |
| **Quick hybridization solution :** | 0.5g BSA, 0.5g PVP, 0.5g Ficoll, 1g SDS, 1g sodium pyrophosphate, 250ml 20X SSC, 100mg denatured salmon sperm DNA in 1l |
| **Prehybridization solution :** | 10% SDS, 7% PEG 6000, 10mg/ml denatured salmon sperm DNA |
| **10X TBE buffer :** | 1M Tris borate, 20mM EDTA (pH8.3) |
| **Stop buffer :** | 10mM NaOH, 95% Formamide, 0.1% Bromophenol blue, 0.1% Xylene cyanol |

## 4. DNA extraction and Purification.

Relatives considered likely to provide information useful for linkage or p53 mutation analysis were contacted by letter after permission was granted by their general practitioners. Twenty-five ml of blood were donated by venesection in heparinised tubes from those willing to assist. Lymphocytes from 10ml of blood were transformed with Epstein Barr virus to create lymphoblastoid cell lines in accordance with standard procedure (Diehl *et al.*, 1964). In many instances these provide a renewable source of constitutional DNA. This procedure was carried out by Dr. C.M. Steel, A. Lennon and Elizabeth Harvey of the MRC Human Genetics Unit.

Chromosome studies were undertaken on 5ml of blood. Peripheral blood lymphocytes were stimulated and high resolution banding performed.

### a) Blood

Extraction and purification of DNA from fresh peripheral blood leukocytes was performed by means of the following method (Boyum, 1968; Gross-Bellard *et al.*, 1972).

To 10ml whole blood, 8ml lysis buffer, 2ml 20% SDS and 20ml phenol were added, mixed and centrifuged (3000 rpm for 10 minutes). To the upper aqueous layer, 10 ml 7.5M ammonium acetate and 40ml isopropylalcohol were added and the solution frozen at -40°C for 30 minutes. DNA was spooled and dried under

vacuum. 10ml resuspension buffer and 100 micrograms RNAase were added and the solution incubated in a waterbath (37°C for 30 minutes). 100µl 20% SDS and 1mg Proteinase K were added followed by incubation in a waterbath (37°C for 8 hours). In this solution was mixed an equal volume of phenol and centrifuged (3000 rpm for 10 minutes). 5ml phenol and 5ml chloroform : iso amylalcohol (24:1) were added to the supernatant and centrifuged (3000 rpm for 10 minutes). To the supernatant 5ml of 7.5M ammonium acetate and 20ml of 100% ethanol were added and the mixture frozen at -40°C for 1 hour. DNA was spooled, dried under vacuum, resuspended in 500µl T.E. buffer and stored at 4°C.

**b) Paraffin embedded tissue**

In a few cases, archival material embedded in paraffin wax was utilised to prepare DNA from deceased or inaccessible individuals (MacDonald and Cohen, 1990). In these instances, care was taken to define non tumour tissue such as uninvolved lymph node, from which DNA was then extracted by means of the following protocol:

Blades, tweezers and other equipment were cleaned with xylene before and between each separate tissue sample mounting. Excess paraffin was trimmed from the tissue and the block mounted on a microtome. From one to ten 10µm sections were cut depending on nuclear density in the section. Sections were lifted into 1.5ml eppendorf tubes with 1ml xylene, mixed for 30 minutes and pelleted by centrifugation (14,000 rpm for 30 seconds). Xylene was

then removed from each tube with a fine-tipped pastette. A further 1ml Xylene was added, mixed, tissue pelleted and xylene removed as before. 0.5ml 100% ethanol was then added, mixed, tissue pelleted and ethanol removed and this process was repeated with the same volume of fresh ethanol. The sample was dried under vacuum, 200µl digestion buffer and 40µg Proteinase K were added and incubated in a 37°C waterbath for 8 hours followed by centrifugation (14,000 rpm for 30 seconds) and stored at 4°C. The DNA present in the aqueous layer was of adequate quality for amplification of genomic fragments by means of polymerase chain reaction (Mullis and Faloona, 1987; Goudie, 1989).

**c) Quantification of extracted DNA.**

The amount of DNA extracted by these methods was calculated in the following way ;

10µl sample was mixed with 740µl distilled water and pipetted into a glass spectrophotometer cell. Absorbance of light at 260nm and 280nm was measured. Pure DNA gave a ratio $^{A260}/_{A280} = 1.8$. The amount of DNA (ng/µl) was calculated by multiplying A260 by a factor of 3750 since absorbance at this wavelength is directly proportional to DNA concentration.

## 5. Allele Data for Linkage Analysis

### a) Restriction fragment length polymorphisms (RFLPs).

These mapped genetic markers are DNA fragments inherited as single copies and which demonstrate allelic variation. Most of the DNA sequence variations initially identified were due to base pair changes that created or destroyed a cleavage site for a specific restriction enzyme, causing a change in length of DNA fragment (RFLP). A marker locus based on a single such variant will have only two alleles (a single cleavage site is either absent or present). This results in limited allele data for linkage analysis purposes (Nakamura *et al.*, 1987).

#### i) Variable number of tandem repeats (VNTRs).

In recent years, polymorphisms with highly variable restriction fragment lengths have been discovered. DNA base sequences of these hypervariable loci indicate that the restriction fragments contain variable number of tandem repeats (VNTRs) of a short (11 to 60 base pairs) oligonucleotide sequence. These can be highly informative in linkage analysis when they exhibit multiple alleles. YNZ 22.1 and CMM86 are such VNTR markers (Nakamura *et al.*, 1987, 1988b). Probe YNZ 22.1 denotes locus D17S30 and maps to chromosome band 17p13.3. Probe CMM86 denotes locus D17S74 and maps to chromosome bands 17q23 (Solomon and Ledbetter 1991, Hall *et al.*, 1992). YNZ 22.1 is a Bam H1 fragment from the cosmid isolated by a zeta-globin oligonucleotide (MacKay, 1988).

CMM86 is a Hinf I /Taq I fragment, 1.0 - 3.5kb in size isolated from the vector pUC18 (Nakamura *et al.*, 1988b).

**(ii) DNA digestion.**

10µg genomic DNA, 4µl Taq1 restriction enzyme buffer and 2µl (10 units) Taq 1 restriction endonuclease were mixed with distilled water to volume 40µl and centrifuged (14,000 rpm for 30 seconds), followed by incubation in a 65°C waterbath for 8 hours and further brief centrifugation. 10µl running buffer was added to make a final volume of 50µl.

**(iii) Agarose gel electrophoresis.** (McDonnel *et al.*, 1977; Southern, 1979).

The electrophoresis tank was prepared and gel tray made flat by means of a spirit level. Autoclave tape was applied to both ends of the tray and a comb inserted with an appropriate number of slots. 4g purified agarose was dissolved in 400ml 1x TAE buffer. Flask, stirrer, agarose and buffer were weighed and the 1% agarose gel boiled. Distilled water was supplemented to regain the original weight and the gel cooled to 60°C with stirring. This was then poured into the electrophoresis tray and trapped air bubbles immediately removed with a pastette, leaving the gel to set completely. The autoclave tape was then removed and a sufficient volume of 1X TAE buffer poured into the tank to completely submerge the gel. The comb was carefully removed and one sample of digested DNA loaded with running buffer into each well. 1X TAE buffer was circulated through the tank and an ice-box by

means of a pump. The tank was connected to a D.C. power pack and run at 60V for 18-24 hours from cathode to anode. One corner of the gel was marked for later orientation and then immersed in 1 litre 1X TAE, to which 50µl 10mg/ml ethidium bromide was added to a final concentration of 0.5µg/ ml. The gel in TAE was gently agitated for 30 minutes, destained with 1 litre distilled water for 10 minutes, placed on a U.V. transilluminator to detect incomplete digestion of DNA and photographed. DNA in the gel was denatured by immersion in 1.25 litres denaturing solution with gentle agitation for 45 minutes. It was then neutralized by immersion in 1.25 litres neutralizing solution with gentle agitation for 1 hour.

**(iv) Southern blotting.** (Southern 1975)

The apparatus was prepared by resting a glass plate (0.5 x 23 x 48cm) on top of a plastic tray (30 x 45cm) containing 2 litres 20X SSC buffer. Whatman 17MM filter paper was placed on this plate with both ends immersed in buffer. Whatman 3MM paper was placed on top of this. Both layers were thoroughly soaked in buffer and air bubbles removed. The gel was removed from neutralizing solution and set on top of the 3MM filter paper and cling-film was used to seal the tray around the gel to prevent evaporation of buffer. A nylon membrane (Hybond N, Amersham, 20 x 23cm) was placed directly over the gel and air bubbles removed. Two pieces of dry 3MM paper were placed over the membrane followed by a large pile of towels (Kleenex) and then green paper towels. A heavy weight was applied to compress the apparatus for 8 hours.

The position of gel wells were marked on the membrane and a corner cut for orientation. The layers down to the gel were then discarded but the remainder of the blotting apparatus was recovered. The membrane was placed on a clean U.V. transilluminator and the side opposed to the gel illuminated to covalently bind DNA to the membrane.

**(v) Hybridization.**

The membrane was placed between two pieces of nylon filter in a tray of 1 litre 2X SSC buffer, rolled-up with DNA side outermost and placed in a glass tube with water-tight stopper. If the probe to be used was YNZ 22.1, 10ml quick-hybridization solution was added. If the probe was CMM86, 10ml prehybridization solution was used. Incubation followed in a rotating drum oven at 65°C for 2 hours.

Labelling of DNA was achieved through the hexamer random priming method (Jacobsen *et al.*, 1974; Feinberg and Vogelstein, 1983) using a Multiple DNA labelling kit (Amersham RPN 1600Y). 4µl of each unlabelled nucleotide in the kit (dTTP, dGTP and dATP) was added to 5µl reaction buffer (tris-HCl pH7.8, 50mM MgCl2 and 100mM 2-mercaptoethanol) and 5µl primer solution (containing nuclease-free bovine serum albumin and random hexanucleotides). 25ng YNZ 22.1 or 200ng CMM86 was boiled at 100°C for 10 minutes then added to the above mixture and centrifuged at 14,000 rpm for 30 seconds. 30µCi [$^{32}$P] of dCTP and 5µl of kit DNA polymerase 1 (klenow fragment in

50mM potassium phosphate at pH 6.5, 10mM 2-mercaptoethanol and 50% glycerol) were also added. The 1.5ml eppendorf tube containing this mixture was incubated in a lead container immersed at 37°C for 2 hours. Addition of 20µl 10mg/ml salmon sperm DNA was then followed by half volume 3M sodium acetate and excess (250µl) 100% ethanol and freezing at -40°C for 30 minutes in order to precipitate the DNA. Depending on which labelled probe was to be hybridized, one of the following protocols was observed.

For YNZ 22.1, the tube was centrifuged at 14,000 rpm for 30 minutes to pellet the labelled probe and 250µl 10mg/ml denatured salmon sperm DNA with excess (100µl) 10µg/ml denatured human placental DNA added. The sample was boiled at 100°C for 10 minutes, centrifuged at 14,000 rpm for 30 seconds, pipetted into the glass bottle containing membrane and quick-hybridizing solution, and incubated in the rotating drum oven at 65°C for 8 hours. Excess solution from the glass bottle was discarded and 200µl high stringency 0.1X SSC wash solution poured in to half fill the bottle which was returned to the oven at 65°C for 10 minutes. The solution was again discarded and fresh high-stringency wash added twice more, returning to the oven after each cycle. Finally the membrane was washed in 0.1X SSC buffer by gentle agitation again at 65°C for 10 minutes.

For CMM86, the tube was centrifuged at 14,000 rpm for 30 minutes to pellet the labelled probe and resuspended in 100µl 10mg/ml denatured human placental DNA, 125µl 20X SSC and

175µl distilled water. The sample was boiled at 100°C for 5 minutes and centrifuged again. The pre-hybridization solution from the glass bottle was discarded and 10ml fresh prehybridization solution and labelled precipitated probe added by pipette followed by incubation in the rotating drum oven at 65°C for 8 hours. Prehybridization solution was then discarded and 200ml 2X SSC poured in to half fill the bottle followed by gentle agitation at room temperature for 20 minutes. This solution was discarded and 200ml 2X SSC plus 0.5ml 20% SDS added and gently agitated at room temperature for 20 minutes. Finally the solution was again discarded and the membrane washed in 200ml 0.1X SSC plus 0.5ml 20% SDS with gentle agitation again at 65°C for 30 minutes.

Whichever probe was hybridized, the labelled membrane was dried between two pieces of filter paper and wrapped in cling-film opposed to a clean, dry Whatman 3MM filter paper of same size with excess air removed from the package. Pre-flashed X-AR Kodak Omat film was placed opposed to the DNA side of the membrane in an X-ray cassette at -70°C for 8 hours and developed in a RGII Fuji X-ray film processor. The labelled membrane was then left with a fresh pre-flashed film for a longer period at -70°C and developed in the same way to reveal fainter bands.

The membrane was cleaned of labelled probe by placing it in 400ml of boiling 0.1% SDS and allowing the solution to cool immediately. It was then stored in a sealed polythene bag at 4°C for repeat hybridization if required.

**b) Microsatellites.** (Emery and Rimoin, 1990)

There are up to 100,000 hypervariable length polymorphisms known as microsatellites which are very short sequence elements of the form (dCdA)n (dGdT)n interspersed throughout the human genome and are estimated to occur every 30 - 60 kilobases (Miesfield *et al.*, 1981; Weber and May, 1989). Polymerase chain reaction (Mullis and Faloona, 1987) using single copy primers flanking the CA repeat microsatellite is necessary to demonstrate the length differences which are multiples of two base pairs.

**(i) Primer Preparation**

Oligonucleotide primers were produced by Agnes Gallacher of the MRC Human Genetics Unit in DNA synthesisers (Applied Biosystems 381A and 391A) and stored in 350µl 8M ammonium hydroxide at concentration 1mg/ml. Primers were precipitated by addition of 35µl 3M sodium acetate and 5µl 100% ethanol followed by freezing at -20°C for 30 minutes. Samples were then spun at 14,000 rpm for 30 seconds and the pellet washed twice in 1ml 100% ethanol by inversion. Following another brief spin the sample was dried over vacuum and stored in 200µl T.E. buffer.

**(ii) Microsatellite Amplification.**

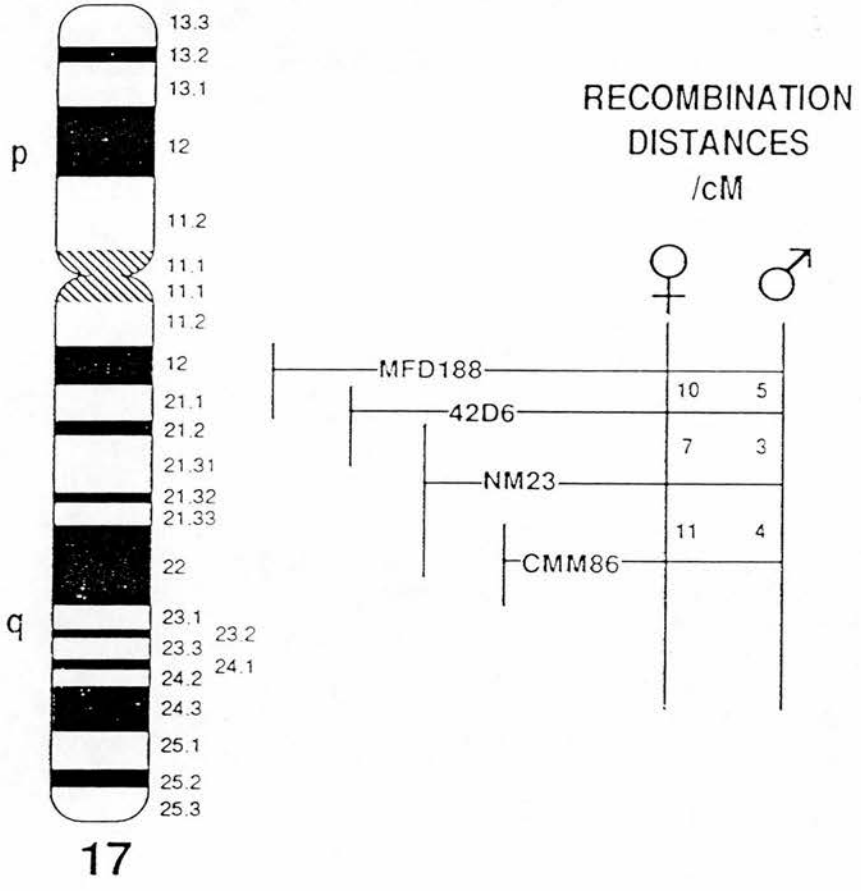
The microsatellites for amplification were nm23 at position 17q21.3-22, 42D6 (D17S588) at 17q21 MFD188 (D17S579) at position 17q12-21 (Hall *et al.*, 1992). For females these are reported as being 11, 18 and 28 cM centromeric to D17S74 respectively (the CMM86 locus). For males these are 4, 7 and 12

cM centromeric to this locus (Easton *et al.*, 1993). These distances are illustrated in Figure 3.

**Figure 3**

Chromosome 17 frame indicating recombination distances between markers CMM86, NM23, 42D6 and MFD188 in males and females (Easton *et al.,* 1993).

Figure 3


13.3
13.2
13.1
p
12
11.2
11.1
11.1
11.2
12
21.1
21.2
21.31
21.32
21.33
22
q
23.1
23.3
23.2
24.2
24.1
24.3
25.1
25.2
25.3
17
RECOMBINATION
DISTANCES
/cM
MFD188
42D6
NM23
CMM86
10
5
7
3
11
4

The process of microsatellite amplification involved mixing of 100ng genomic DNA with 50pmoles of each primer, 25µl (2mM) of each dATP, dGTP, dTTP and dCTP (Boehringer Mannheim), 0.75U Taq1 polymerase and 5µl Taq 1 polymerase 10X reaction buffer (both from Promega Ltd, UK) to make a reaction volume of 50µl. 2 drops of light mineral oil were laid onto each sample to prevent evaporation during temperature cycling. From this sample, nm23 was amplified using primer sequences TTG ACC GGG GTA GAG AAC TC and TCT CAG TAC TTC CCG TGA CC. 42D6 was amplified using primer sequences CCT GGT CTA GGA AGA GTG TCA and GTG TAA GCA TCT GTG TAT ACT AC. MFD188 was amplified using primer sequences AGT CCT GTA GAC AAA ACC TG and CAG TTT CAT ACC AAG TTC CT. Amplification was achieved by means of automated thermocyclers (Biometra TRIO-thermoblock). For both nm23 and MFD188 PCR conditions were 94°C 5 minutes followed by 30 cycles of 94°C 1 minute, 55°C 1 minute and 72°C 1 minute with a final 5 minute extension at 72°C. Conditions for 42D6 were 27 cycles of 94°C 30 seconds, 55°C 30 seconds and 72°C 1 minute.

To determine whether PCR amplification had been successfully achieved, a 2% agarose gel of volume 100ml was made (2g purified agarose in 100ml 1x TAE buffer) and allowed to set in a small gel tray (11 x 14cm). 10µl of amplified PCR product with 2.5µl running buffer were loaded into each well. The tray was immersed in a solution of 1X TAE buffer containing 25µl 10mg/ml ethidium bromide and run at 100V for 90 minutes. After de-staining with

500ml distilled water, a U.V. transilluminator (UVP inc.) was employed to identify amplified microsatellite fragments migrating from cathode to anode.

**(iii) Polyacrylamide gel electrophoresis.**

Polymorphisms in microsatellite markers of different lengths were separated by means of polyacrylamide gel electrophoresis for which the following method was used :-

Glass plates (30 x 30cm) were successively cleaned in detergent, tap water and distilled water. The inside surface of each plate was washed with 100% ethanol, dried and coated with dimethyldichlorosilane solution to endow water-repellent properties. The two inside surfaces were opposed but separated by 0.75mm with spacers. The edges of the two plates were sealed firmly together with autoclave tape. The apparatus was plugged at the base by 6ml of 10% acrylamide mixture containing 1.25ml 40% acrylamide, 4.75ml distilled water, 1mg ammonium persulphate and 12µl TEMED (N,N,N',N', tetramethylethylenediamine). A 10% acrylamide solution with denaturant properties was made by mixing 37.5ml 40% acrylamide and 54g urea with 7.5ml 20X TBE buffer and adding distilled water to a final volume of 150ml. After addition of 25mg ammonium persulphate and 150µl TEMED this mixture was poured between the plates and a comb inserted by the straight edge into the solution which was left for 30 minutes until polymerisation. The comb and tape were then removed and after washing in distilled water and 1X TBE buffer, the comb was re-

inserted with teeth into the gel. The glass plates sandwiching the gel were then placed in an electrophoresis tank (Bethesda Research Laboratories Life Technologies inc., model S2) and clampled together. 500ml 1X TBE was poured in to each of the upper and lower tank reservoirs. The comb was removed from the gel and the wells washed in 1X TBE buffer.

PCR products were prepared by mixing 14µl sample with 8µl stop buffer, heated at 80°C for 3 minutes and cooled on ice. The samples were loaded and the gel run at 1500 - 1900V, 30 - 45mA (60W) until bromophenol blue dye from the stop buffer ran out of the gel (approx. 3 hours). The gel was then soaked in 500ml solution of 10 - 12% methanol and 10% glacial acetic acid for 2 hours. This process was repeated using fresh solutions. The gel was dried in an oven at 70°C for 8 hours and cooled to room temperature.

**(iv) DNA electroblotting and hybridization with radiolabelled (dCdA) probe**

DNA fragments were transferred from the relevant part of the gel to the nylon blotting membrane, Hybond N (Amersham International plc.) by means of standard electroblotting procedures (Stellwag and Dahlberg, 1980). Hybridization of CA repeat elements on the membrane to a radio-labelled (CA)11 repeat probe was achieved and photographed in the standard way (Cohen *et al.*, 1992) Electroblotting, hybridization and autoradiography were carried out

by Dr Brian Cohen and Mairi Wallace at the MRC Human Genetics Unit.

## 6. p53 Mutation Analysis.

### (a) Denaturing gradient gel electrophoresis (DGGE). (Fisher and Lerman, 1983)

Germline mutations in the normal wild-type sequence of the p53 gene such as small base pair substitutions can be responsible for malignant predisposition (Soussi and De Fromental, 1992). These changes can be detected by Denaturing Gradient Gel Electrophoresis (DGGE). The DGGE separation technique relies on the fact that strand dissociation of amplified DNA fragments in discrete sequence-dependent melting domains cause an abrupt decrease in mobility visible on an acrylamide gel containing a gradient of denaturant (Borresen *et al.*, 1991). After PCR amplification up to four fragments appear in lanes in which a single base pair mismatch resulting from a mutation in the p53 gene produces two homoduplexes (wild-type double strand and mutant double strand) and two heteroduplexes (the two different arrangements of mutant and wild-type DNA in double strand formation) (Erlich, 1989).

#### i) Primer preparation.

Oligonucleotide primers were produced in the same way as described in section 5 (b) (i)

**(ii) Exon amplification**

Primer sequences were manufactured which would enable amplification of the highly conserved domains of exons 5, 7 and 8 of the p53 gene (Borresen *et al.*, 1991). The codons covered by four sets of primers are responsible for 20 of the 25 reports of germ-line p53 mutations published to date (Santibanez-Koref *et al.*, 1991a, 1991b; De Fromental and Soussi, 1992; Prosser *et al.*, 1992; Iavarone *et al.*, 1992; Sidransky *et al.*, 1992b).

GC clamps were attached to primers (Erlich, 1989). Primer sequences TTC CTC TTC CTG CAG TAC TC and CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTG GCG CGG ACG CGG GTG CCG amplified codons 128 - 153 of exon 5. Sequences CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTT CCA CAC CCC CGC CCG GCA and GCC CCA GCT GCT CAC CAT CG amplified codons 155 - 185 of exon 5. Sequences CAC CAT CCA CTA CAA CTA CA and GCG GGC GGC GCG GGG CGC GGG CAG GGC GGC GGG GGC GGG CAG TCT TCC AGT GTG ATG ATG amplified codons 237 - 253 of exon 7. Sequences ATC CTG AGT AGT GGT AAT CT and GCG GGC GGC GCG GGG CGC GGG CAG GGC GGC GGG GGC GGG CTA CCT CGC TTA GTG CTC CCT amplified codons 265 - 301 of exon 8. Blood lymphocyte DNA from subjects under analysis were mixed with primer pairs, nucleotides, Taq 1 polymerase and buffer as described in section 5(b)(ii). PCR conditions were 94°C for 7 minutes followed by 35

cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds and ended with a final 10 minute extension at 72°C.

**(iii) Polyacrylamide gel electrophoresis and staining.**

Perpendicular gradient denaturing gels (18 x 15 x 0.1cm) containing TEMED (10µl/gel), ammonium persulphate (5mg/gel) and a 10% acrylamide solution in 1x TBE buffer were cast with a 2-channel pump mixing 80% denaturant solution into 20% solution to create a 30 - 80% denaturant gradient (100% denaturant corresponds to 7M urea and 40% formamide. PCR products were loaded into wells along the top of the gel and run across 80V with the electrophoresis direction parallel to the denaturant gradient. Gels were submerged in 1X TBE buffer at 56°C in a self-constructed cell based on protocol criteria developed by Borresen *et al.*, (1991). These modifications allowed the glass plates surrounding the gels to be in direct contact with the buffer on both sides. Extensive circulation of buffer was provided during the runs. Running time was 2 - 3 hours. After electrophoresis, the gels were stained in ethidium bromide (2mg/l TBE) and photographed using a U.V. transilluminator.

**b) Sequencing.**

The polymerised samples which produced from two to four bands on DGGE (indicating non-identical p53 strands) were cut-out of the gel. Each excised band was melted and DNA sequenced using dideoxynucleotide labelling within the sequenase DNA sequencing kit and run on a gradient denaturing gel which was radiolabelled

and autoradiographed. Sequencing was performed by Dr Brian Cohen and Mairi Wallace of the MRC Human Genetics Unit.

## 7. Linkage analysis.

We have used the program for pedigree analysis MENDEL (Lange *et al.*, 1988). Within this program we have utilised the segregation analysis model determined by Iselius *et al.*, (1991) in which 1248 nuclear families with breast cancer were collected from two British series. This contrasts with the Cancer and Steroid Hormone Study (CASH) analysis by Claus et al., (1991) in which four times this number of nuclear families were utilised, but from centres only in the U.S.A. In the British model the estimate of gene frequency is 0.003 with lifetime penetrance of 0.83 compared with 0.92 in the CASH study. In place of liability classes of risk of breast cancer by age-group, mathematical formulae were inserted into the program. These formulae described best-fit curves for breast cancer risk by age for gene carriers and non-gene carriers as estimated by Iselius *et al.*, (1991).

For female (carriers) heterozygotes:

$$\text{Probability}\,(T \leq t) = \frac{0.9}{1 + [35/t - 15]^3}$$

For female (non-carriers) homozygotes:

$$\text{Probability } (T \leq t) = \frac{0.1}{1 + [50 / t - 15]^4}$$

t = age of individual

Probability ($T \leq t$) = probability of developing breast cancer prior to age t.

In tabulated form these equations produce estimates for probability of developing breast cancer which are illustrated below :

| | **Probability of developing breast cancer** | |
|---|---|---|
| **Age** | **Heterozygotes (carriers)** | **Homozygotes (non-carriers)** |
| < 25 | 0.021 | 0.0002 |
| < 30 | 0.066 | 0.001 |
| < 40 | 0.240 | 0.006 |
| < 50 | 0.450 | 0.019 |
| < 60 | 0.612 | 0.040 |
| < 70 | 0.716 | 0.059 |
| < 80 | 0.778 | 0.074 |

For the purpose of the model, male breast cancer at any age was defined as having an incidence of 1% that of female breast cancer at the same age, but otherwise followed the same age incidence

curves as female breast cancer (Lynch and Kullander, 1987a). In a separate analysis the above conditions were maintained but in addition ovarian cancer at any age was defined as endowing the patient with a likelihood of carrying the gene equivalent to that for female breast cancer at age 25, with and without a similar assumption about ovarian cancer. The model requires an estimate of the probabilty that an affected individual is either a gene carrier or non-gene carrier. The probability estimates of Iselius et al., (1991) are utalised in this programme. Thus an affected individual belonging to a low risk liability class (an older woman) is less likely to have the putative breast cancer gene than an affected individual in a high-risk liability class (a younger woman). The probability of an affected individual being a gene carrier is tabulated below :

| Age | Probability of affected individual being a gene carrier. |
|---|---|
| 20 - 24 | 0.897 |
| 25 - 29 | 0.833 |
| 30 - 39 | 0.405 |
| 40 - 49 | 0.202 |
| 50 - 59 | 0.138 |
| 60 - 69 | 0.109 |
| 70 - 79 | 0.097 |
| > 80 | 0.076 |

Observations on allele segregation in cases of colon cancer and other malignancies were made in order to investigate the possibility

of linkage to a breast cancer gene in these diseases also. The analysis required an estimate of population frequency of alleles for each polymorphic marker analysed. These were determined from DNA analysis of spouses of breast cancer family members. These non-blood relatives provided upwards of 132 individuals from which population allele frequencies were estimated. Relative allele frequencies of all markers investigated are illustrated in Table I.

**Table I**

Population of allele frequencies for YNZ22.1, CMM86, NM23, 42D6 and MFD188.

**Table I**

| Allele Number | Relative Frequency of Alleles (%) | | | | |
|---|---|---|---|---|---|
| | YNZ22.1 | CMM86 | NM23 | 42D6 | MFD188 |
| 1 | 10.6 | 1.5 | 35.9 | 2.0 | 0.7 |
| 2 | 11.4 | 7.2 | 9.0 | 8.7 | 2.1 |
| 3 | 20.5 | 31.9 | 12.4 | 2.0 | 4.8 |
| 4 | 30.3 | 18.8 | 22.5 | 0.7 | 6.9 |
| 5 | 17.4 | 6.5 | 20.2 | 1.7 | 15.9 |
| 6 | 5.3 | 5.8 | | 1.7 | 22.0 |
| 7 | 4.5 | 13.0 | | 14.1 | 14.5 |
| 8 | | 11.6 | | 8.1 | 4.2 |
| 9 | | 1.5 | | 11.4 | 6.2 |
| 10 | | 2.2 | | 19.5 | 4.1 |
| 11 | | | | 24.1 | 12.4 |
| 12 | | | | 6.0 | 6.2 |
| **Total** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** |

## 8. Structural Chromosome Analysis.

Blood samples were obtained by venesection from both affected and unaffected relatives. Chromosome studies were undertaken on 5ml blood. 0.8ml aliquots of whole blood were cultured in a variety of different media including DF10 (a low folate medium for expression of folate sensitive fragile sites) and RPMI 1640 Dutch modification using thymidine ($4 \times 10^{-7}$m) for thymidylate stress. All cultures were supplemented with 10% foetal calf serum, penicillin and streptomycin. Lymphocytes were stimulated with phytohaemagluttinin and high resolution banding was carried out by synchronisation of cell division in culture with either bromodeoxyuridine, or methotrexate and harvest of the cells 5 hours after the release of the block and arrest of the cells with colcemid (1μg/ml) for 10 minutes (Yunis, 1976). At least 10 nuclei were analysed from each individual. This laboratory work was carried out by Dr G. Spowart and H. Morrison of the MRC Human Genetics Unit. Quantitative differences in the proportion of cells with rearrangements could be compared in individuals who were identified as either carriers or non carriers of a 17q breast cancer susceptibility gene and assessed statistically through the chi-square method.

# RESULTS

1. **p53 mutation analysis in breast cancer families.**

2. **Linkage analysis.**

   i) Two point LOD scores.

   ii) Identification of probable 17q breast cancer gene carriers.

   iii) Penetrance, age-incidence and survival in 17q breast cancer gene carriers.

3. **Structural chromosome analysis.**

## Results

### 1. p53 mutation analysis in breast cancer families.

A number of individuals in two breast cancer pedigrees illustrated in Figure 2 were screened for germline p53 mutation. By means of DGGE and gene sequencing, three individuals in pedigree A were shown to exhibit an exon 8 mutation at codon 267 in one copy of p53; CGG to CAG thereby replacing arginine with glutamine (Prosser *et al.*, 1992). The mutation lies in the general region of previously reported mutations (De Fromental and Soussi, 1992). More specifically it is situated between two evolutionarily conserved zones within an arginine codon invariant in all species studied (Prosser *et al.*, 1992). From analysis of pedigree A it can be inferred that all breast cancer cases and in addition the individual with lung and cervical cancer must also have inherited this mutation. Three individuals in pedigree A were screened and found to have only wild types p53 copies. One further distant relative not illustrated in pedigree A who developed a primary brain tumour aged 35 was also found to have wild type p53. He was, however, separated from the closest cancer case in pedigree A by four generations.

DGGE and gene sequencing in members of pedigree B have revealed a germline exon 8 mutation at codon 273 of the p53 gene (CGT to CAT; arginine to histidine) in a deceased individual diagnosed with breast cancer at age 33 and in her daughter with osteosarcoma at age

17. Two unaffected brothers of the proband were found to have wild type germline p53.

## 2. Linkage analysis

### i) Two point LOD scores :

Table II records the total number of affected individuals with breast or ovarian cancer in pedigrees typed at markers located on chromosome 17. The number of individuals for whom lymphocyte DNA or paraffin-wax embedded material was utilised for genotyping is also shown, together with typing success rates.

*17p analysis* :

Table III records LOD scores at YNZ 22.1 in 5 breast cancer pedigrees.

*17q analysis :*

Two point LOD scores for separate markers on chromosome 17q are recorded in Tables IV, V, VI and VII. Pedigrees are ranked according to average age of onset of breast cancer in the family. Scores in this analysis are calculated via a model which assumes that the disease-associated gene causes breast cancer alone; both unilateral and bilateral disease are assumed to follow the standard model age-incidence curves predicted by segregation analysis studies.

**Table II**

Number of cases of unilateral and bilateral breast cancer and ovarian cancer in 15 Edinburgh pedigrees subjected to linkage analysis. Number of blood and wax-section DNA samples available and number of individuals successfully typed at one or more loci are also shown.

## Table II

| Pedigree ID | Number of cases | | | Blood DNA | | Wax sections | |
|---|---|---|---|---|---|---|---|
| | Unilateral breast cancer | Bilateral breast cancer | Ovarian cancer | Number of samples | Typing success | Number of sections | Typing success |
| 1 | 5 | 3 | 1 | 49 | 49 | 8 | 8 |
| 2 | 5 | 3 | 3 | 23 | 23 | 3 | 3 |
| 3 | 3 | 1 | 0 | 9 | 9 | 0 | 0 |
| 11 | 8 | 0 | 0 | 27 | 26 | 3 | 3 |
| 12 | 5 | 0 | 0 | 13 | 13 | 2 | 2 |
| 16 | 4 | 1 | 0 | 8 | 8 | 4 | 4 |
| 27 | 7 | 0 | 0 | 8 | 8 | 0 | 0 |
| 29 | 4 | 0 | 0 | 8 | 8 | 8 | 8 |
| 30 | 3 | 2 | 0 | 9 | 9 | 2 | 2 |
| 33 | 3 | 0 | 0 | 4 | 4 | 2 | 2 |
| 37 | 2 | 3 | 2 | 6 | 6 | 2 | 2 |
| 38 | 3 | 0 | 0 | 15 | 15 | 2 | 2 |
| 84 | 3 | 0 | 0 | 3 | 3 | 2 | 2 |
| 1021 | 3 | 1 | 1 | 15 | 15 | 0 | 0 |
| 2000 | 2 | 1 | 3 | 9 | 9 | 0 | 0 |
| **Total** | **60** | **15** | **10** | **206** | **205** | **38** | **38** |

**Table III - VII**

Two-point LOD scores at separate markers on chromosome 17 under assumption that all breast cancer conforms to model curves.

**Table III :** YNZ22.1

**Table IV :** CMM86

**Table V :** NM23

**Table VI :** 42D6

**Table VII :** MFD188

Table III

| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | |
|---|---|---|---|---|---|---|---|
| | | **0.3** | **0.2** | **0.1** | **0.05** | **0.01** | **0.001** |
| 1 | 37.6 | 0.45 | 0.81 | 1.16 | 1.32 | 1.45 | 1.48 |
| 11 | 43.4 | 0.01 | 0.02 | 0.04 | 0.06 | 0.07 | 0.08 |
| 12 | 45.7 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 |
| 2 | 46.0 | -0.05 | -0.12 | -0.28 | -0.46 | -0.79 | -0.94 |
| 3 | 52.5 | 0.07 | 0.01 | -0.18 | -0.41 | -0.92 | -1.27 |
| **Total** | | **0.48** | **0.72** | **0.74** | **0.51** | **-0.19** | **-0.65** |

Table IV

| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0.40** | **0.30** | **0.20** | **0.10** | **0.075** | **0.050** | **0.025** | **0.000001** |
| 1 | 37.6 | 0.02 | 0.05 | 0.06 | -0.05 | -0.12 | -0.24 | -0.45 | -1.11 |
| 11 | 43.4 | 0.01 | 0.02 | 0.06 | 0.08 | 0.09 | 0.10 | 0.11 | 0.12 |
| 12 | 45.7 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2 | 46.0 | -0.02 | -0.03 | -0.05 | -0.07 | -0.08 | -0.09 | -0.10 | -0.10 |
| 3 | 52.5 | 0.00 | 0.02 | 0.04 | 0.09 | 0.10 | 0.12 | 0.13 | 0.15 |
| **Total** | | **0.01** | **0.06** | **0.10** | **0.03** | **-0.01** | **-0.11** | **-0.31** | **-0.94** |

Table V

| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0.40** | **0.30** | **0.20** | **0.10** | **0.075** | **0.050** | **0.025** | **0.000001** |
| 1 | 37.6 | 0.26 | 0.56 | 0.85 | 1.11 | 1.17 | 1.23 | 1.28 | 1.34 |
| 11 | 43.4 | 0.01 | 0.02 | 0.04 | 0.06 | 0.06 | 0.07 | 0.07 | 0.07 |
| 16 | 45.4 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 12 | 45.7 | -0.00 | -0.00 | -0.01 | -0.01 | -0.01 | -0.01 | -0.01 | -0.02 |
| 2 | 46.0 | 0.01 | 0.03 | 0.10 | 0.21 | 0.24 | 0.28 | 0.32 | 0.36 |
| 3 | 52.5 | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.02 |
| 1021 | 56.0 | -0.00 | -0.01 | -0.02 | -0.04 | -0.04 | -0.05 | -0.05 | -0.06 |
| **Total** | | **0.27** | **0.61** | **0.98** | **1.35** | **1.44** | **1.55** | **1.64** | **1.72** |

Table VI

| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0.40** | **0.30** | **0.20** | **0.10** | **0.075** | **0.050** | **0.025** | **0.000001** |
| 37 | 36.2 | 0.02 | 0.09 | 0.21 | 0.35 | 0.39 | 0.42 | 0.46 | 0.50 |
| 1 | 37.6 | -0.01 | 0.07 | 0.18 | 0.20 | 0.16 | 0.08 | -0.09 | -0.66 |
| 11 | 43.4 | 0.23 | 0.63 | 1.11 | 1.60 | 1.73 | 1.85 | 1.96 | 2.08 |
| 2000 | 43.7 | 0.00 | 0.01 | 0.02 | 0.04 | 0.04 | 0.05 | 0.05 | 0.06 |
| 38 | 44.7 | -0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 16 | 45.4 | 0.05 | 0.17 | 0.32 | 0.51 | 0.55 | 0.60 | 0.64 | 0.68 |
| 33 | 45.7 | 0.01 | 0.05 | 0.11 | 0.17 | 0.19 | 0.21 | 0.23 | 0.24 |
| 12 | 45.7 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 |
| 2 | 46.0 | 0.04 | 0.20 | 0.42 | 0.59 | 0.60 | 0.58 | 0.49 | 0.22 |
| 29 | 46.5 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 |
| 27 | 52.5 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 3 | 52.5 | 0.02 | 0.08 | 0.20 | 0.38 | 0.43 | 0.48 | 0.53 | 0.58 |
| 1021 | 56.0 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 |
| 30 | 58.4 | 0.02 | 0.05 | 0.11 | 0.19 | 0.21 | 0.24 | 0.27 | 0.30 |
| 84 | 60.7 | -0.00 | -0.01 | -0.03 | -0.05 | -0.05 | -0.05 | -0.05 | -0.06 |
| **Total** | | **0.37** | **1.34** | **2.64** | **3.96** | **4.22** | **4.41** | **4.45** | **3.90** |

Table VII

| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **0.40** | **0.30** | **0.20** | **0.10** | **0.075** | **0.050** | **0.025** | **0.000001** |
| 37 | 36.2 | 0.03 | 0.11 | 0.25 | 0.41 | 0.46 | 0.50 | 0.54 | 0.59 |
| 1 | 37.6 | 0.06 | 0.20 | 0.34 | 0.39 | 0.38 | 0.35 | 0.29 | 0.18 |
| 11 | 43.4 | 0.14 | 0.20 | 0.16 | -0.09 | -0.22 | -0.39 | -0.67 | -1.41 |
| 2000 | 43.7 | 0.01 | 0.01 | 0.01 | 0.03 | 0.03 | 0.04 | 0.04 | 0.05 |
| 38 | 44.7 | -0.00 | -0.00 | -0.00 | -0.01 | -0.01 | -0.01 | -0.01 | -0.01 |
| 16 | 45.4 | -0.03 | -0.08 | -0.17 | -0.33 | -0.38 | -0.45 | -0.53 | -0.62 |
| 12 | 45.7 | -0.00 | -0.00 | -0.01 | -0.02 | -0.02 | -0.02 | -0.02 | -0.03 |
| 2 | 46.0 | 0.04 | 0.16 | 0.41 | 0.77 | 0.87 | 0.97 | 1.08 | 1.18 |
| 29 | 46.5 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 27 | 52.5 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 |
| 1021 | 56.0 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 |
| 30 | 58.4 | 0.02 | 0.09 | 0.23 | 0.43 | 0.49 | 0.55 | 0.61 | 0.67 |
| 84 | 60.7 | -0.00 | -0.01 | -0.01 | -0.02 | -0.02 | -0.03 | -0.03 | -0.03 |
| **Total** | | **0.26** | **0.68** | **1.19** | **1.56** | **1.56** | **1.50** | **1.28** | **0.56** |

Five families were typed at CMM86. Haplotyping at this locus was limited due to difficulties with hybridisation. As a result, only one pedigree could be considered informative at this locus and the pedigree did not exhibit tight linkage to a gene in close proximity (Table IV). Analysis of allele data from 7 families at NM23 was again largely uninformative, but a positive LOD score in one pedigree suggested that the susceptibility gene might be closer to this locus (Table V). Typing at 42D6 and MFD188 however seemed to provide the greatest evidence for linkage (Tables VI and VII). Therefore most effort was expended in typing alleles at these loci in all available pedigrees. Pedigrees 3 and 33 were omitted from analysis at locus MFD188, due to an inadequate quantity of DNA available for typing from crucial individuals.

Tables VIII, IX and X record LOD scores at the tightly linked markers 42D6 and MFD188 only. On examination of allele segregation within Edinburgh pedigrees, it was apparent that all alleles typed at 42D6 and MFD188 in cases of bilateral breast cancer and ovarian cancer also segregated with other breast cancer or other ovarian cancer cases in the same pedigree. Therefore, in consequence of a high probability of carrier status in bilateral breast cancer and ovarian cancer cases (Easton *et al.*, 1993), scores in Table VIII assume that bilateral breast cancer at any age has endowed the patient with a likelihood of carrying the gene equivalent to that for breast cancer at age 25. This affects LOD scores in 8 out of 15 pedigrees. Scores in Table IX assume that ovarian cancer at any age is equivalent to breast cancer at age 25 with both bilateral and unilateral breast cancer made to

conform to standard model age-incidence curves. This affects LOD scores in 8 out of 15 pedigrees. Scores in Table X assume that both ovarian and bilateral breast disease at any age are equivalent to breast cancer at age 25. Figure 4 records summated 2-point LOD scores from Table X in graphic form.

**Tables VIII → X**

Two-point LOD scores at a) 42D6 and b) MFD188 under assumption that unilateral breast cancer approximates to model curves and that :

**Table VIII :** bilateral disease endows the individual with a likelihood of carrying the gene equivalent to that for unilateral breast cancer at age 25.

**Table IX :** ovarian cancer at any age endows a risk of carrying the gene equivalent to that for unilateral breast cancer at age 25.

**Table X :** either bilateral breast cancer or ovarian cancer at any age endows a risk of carrying the gene equivalent to that for unilateral breast cancer at age 25.

Table VIII

| (a) 42D6 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.02 | 0.10 | 0.22 | 0.36 | 0.40 | 0.44 | 0.48 | 0.51 |
| 1 | 37.6 | -0.01 | 0.08 | 0.19 | 0.22 | 0.19 | 0.11 | -0.06 | -0.63 |
| 2000 | 43.7 | 0.02 | 0.07 | 0.17 | 0.31 | 0.35 | 0.40 | 0.45 | 0.51 |
| 16 | 45.4 | 0.06 | 0.20 | 0.38 | 0.58 | 0.63 | 0.67 | 0.72 | 0.77 |
| 2 | 46.0 | 0.06 | 0.24 | 0.49 | 0.68 | 0.70 | 0.68 | 0.60 | 0.33 |
| 3 | 52.5 | 0.02 | 0.08 | 0.21 | 0.38 | 0.43 | 0.48 | 0.53 | 0.58 |
| 1021 | 56.0 | -0.00 | -0.00 | -0.01 | -0.01 | -0.01 | -0.01 | -0.01 | -0.01 |
| 30 | 58.4 | 0.09 | 0.25 | 0.45 | 0.65 | 0.71 | 0.76 | 0.81 | 0.86 |
| **Total (including other 7 pedigrees typed in Table VI)** | | **0.49** | **1.66** | **3.29** | **4.92** | **5.27** | **5.54** | **5.66** | **5.19** |
| (b) MFD188 | | | | | | | | | |
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.03 | 0.12 | 0.26 | 0.43 | 0.47 | 0.51 | 0.56 | 0.60 |
| 1 | 37.6 | 0.07 | 0.21 | 0.36 | 0.42 | 0.41 | 0.38 | 0.32 | 0.22 |
| 2000 | 43.7 | 0.00 | 0.01 | 0.02 | 0.03 | 0.03 | 0.04 | 0.04 | 0.04 |
| 16 | 45.4 | -0.03 | -0.10 | -0.23 | -0.46 | -0.56 | -0.68 | -0.87 | -1.19 |
| 2 | 46.0 | 0.13 | 0.48 | 0.93 | 1.42 | 1.54 | 1.66 | 1.78 | 1.90 |
| 1021 | 56.0 | -0.00 | -0.00 | -0.00 | -0.00 | -0.00 | -0.01 | -0.01 | -0.01 |
| 30 | 58.4 | 0.12 | 0.38 | 0.71 | 1.05 | 1.13 | 1.21 | 1.29 | 1.37 |
| **Total (including other 6 pedigrees typed in Table VII)** | | **0.46** | **1.28** | **2.18** | **2.75** | **2.77** | **2.68** | **2.40** | **1.47** |

Table IX

| (a) 42D6 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.02 | 0.10 | 0.21 | 0.36 | 0.39 | 0.43 | 0.47 | 0.51 |
| 1 | 37.6 | -0.04 | 0.07 | 0.21 | 0.21 | 0.26 | 0.22 | 0.14 | -0.81 |
| 2000 | 43.7 | 0.05 | 0.17 | 0.32 | 0.49 | 0.54 | 0.58 | 0.62 | 0.66 |
| 2 | 46.0 | 0.11 | 0.33 | 0.57 | 0.72 | 0.73 | 0.70 | 0.61 | 0.39 |
| 1021 | 56.0 | -0.00 | -0.01 | -0.02 | -0.04 | -0.04 | -0.05 | -0.05 | -0.06 |
| **Total (including other 10 pedigrees typed in Table VI)** | | **0.46** | **1.63** | **3.15** | **4.62** | **4.91** | **5.13** | **5.19** | **4.53** |
| **(b) MFD188** | | | | | | | | | |
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.03 | 0.11 | 0.25 | 0.42 | 0.47 | 0.51 | 0.55 | 0.60 |
| 1 | 37.6 | 0.06 | 0.20 | 0.34 | 0.39 | 0.38 | 0.35 | 0.29 | 0.18 |
| 2000 | 43.7 | 0.06 | 0.20 | 0.37 | 0.56 | 0.61 | 0.65 | 0.70 | 0.74 |
| 2 | 46.0 | 0.12 | 0.43 | 0.85 | 1.31 | 1.43 | 1.54 | 1.66 | 1.77 |
| 1021 | 56.0 | -0.00 | -0.01 | -0.01 | -0.01 | -0.02 | -0.02 | -0.02 | -0.03 |
| **Total (including other 8 pedigrees typed in Table VII)** | | **0.40** | **1.14** | **2.01** | **2.65** | **2.72** | **2.71** | **2.54** | **1.87** |

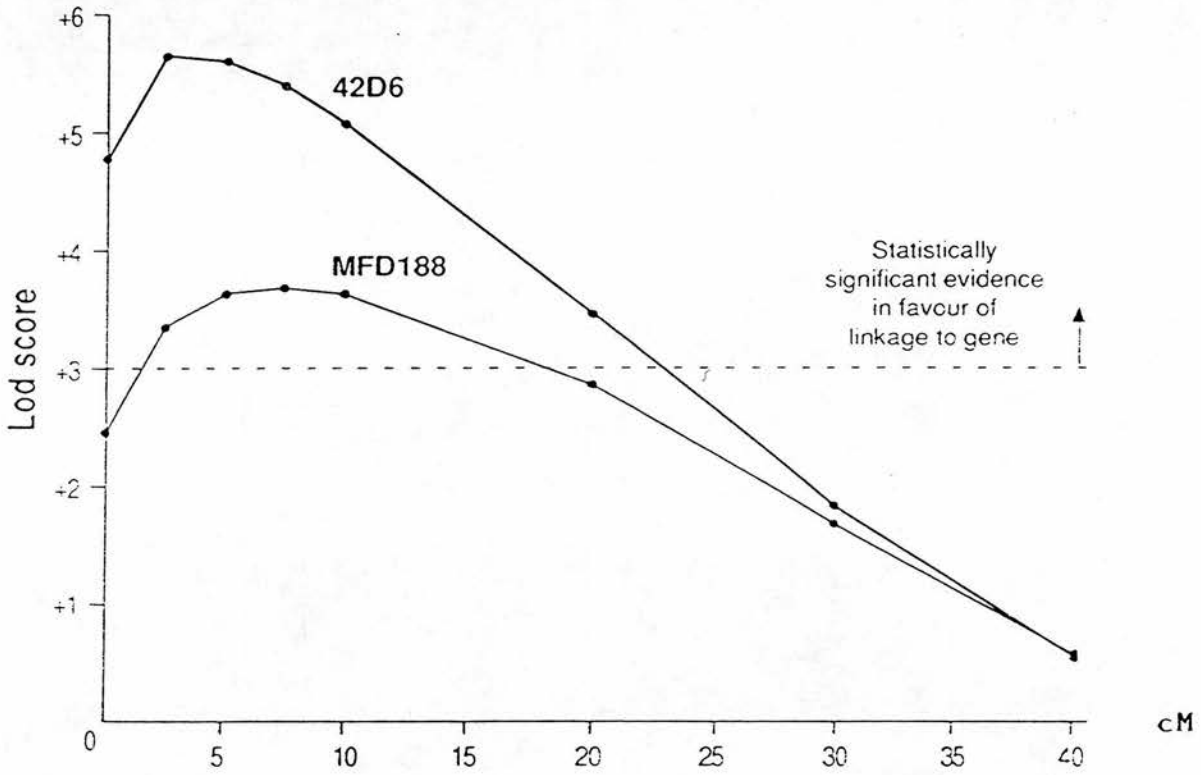
Table X

| (a) 42D6 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.02 | 0.10 | 0.22 | 0.36 | 0.40 | 0.44 | 0.48 | 0.51 |
| 1 | 37.6 | -0.01 | 0.08 | 0.19 | 0.22 | 0.19 | 0.11 | -0.06 | -0.63 |
| 2000 | 43.7 | 0.05 | 0.18 | 0.34 | 0.52 | 0.56 | 0.60 | 0.65 | 0.69 |
| 16 | 45.4 | 0.06 | 0.20 | 0.38 | 0.58 | 0.63 | 0.67 | 0.72 | 0.77 |
| 2 | 46.0 | 0.09 | 0.32 | 0.59 | 0.80 | 0.81 | 0.78 | 0.64 | -0.01 |
| 3 | 52.5 | 0.02 | 0.08 | 0.21 | 0.38 | 0.43 | 0.48 | 0.53 | 0.58 |
| 1021 | 56.0 | -0.01 | -0.05 | -0.11 | -0.20 | -0.23 | -0.26 | -0.29 | -0.32 |
| 30 | 58.4 | 0.09 | 0.25 | 0.45 | 0.65 | 0.71 | 0.76 | 0.81 | 0.86 |
| **Total (including other 7 pedigrees typed in Table VI)** | | **0.54** | **1.82** | **3.46** | **5.05** | **5.36** | **5.59** | **5.62** | **4.71** |
| (b) MFD188 | | | | | | | | | |
| Pedigree ID | Average age at onset of breast cancer | LoD score at theta | | | | | | | |
| | | 0.40 | 0.30 | 0.20 | 0.10 | 0.075 | 0.050 | 0.025 | 0.000001 |
| 37 | 36.2 | 0.03 | 0.12 | 0.26 | 0.43 | 0.47 | 0.51 | 0.56 | 0.60 |
| 1 | 37.6 | 0.07 | 0.21 | 0.36 | 0.42 | 0.41 | 0.38 | 0.32 | 0.21 |
| 2000 | 43.7 | 0.06 | 0.21 | 0.39 | 0.58 | 0.63 | 0.68 | 0.73 | 0.77 |
| 16 | 45.4 | -0.03 | -0.10 | -0.23 | -0.46 | -0.56 | -0.68 | -0.87 | -1.19 |
| 2 | 46.0 | 0.19 | 0.69 | 1.29 | 1.88 | 2.02 | 2.16 | 2.30 | 2.44 |
| 1021 | 56.0 | -0.00 | -0.02 | -0.06 | -0.14 | -0.17 | -0.20 | -0.24 | -0.28 |
| 30 | 58.4 | 0.12 | 0.38 | 0.71 | 1.05 | 1.13 | 1.21 | 1.29 | 1.37 |
| **Total (including other 6 pedigrees typed in Table VII)** | | **0.57** | **1.68** | **2.86** | **3.63** | **3.69** | **3.63** | **3.38** | **2.47** |

**Figure 4**

Summated two-point LOD scores from Table X at markers 42D6 and MFD188 for varying genetic distances from the susceptibility gene illustrated in graphic form.

Figure 4


+6
+5
+4
+3
+2
+1
0
Lod score
42D6
MFD188
Statistically
significant evidence
in favour of
linkage to gene
5
10
15
20
25
30
35
40
cM

An examination of allele segregation with colon cancer was also made. There were 13 cases of colon cancer in the 15 pedigrees. Allele data from these revealed that alleles in 9 colon cancer cases segregated elsewhere in the pedigree with breast cancer whereas in 4 they did not co-segregate in this way. A similar study of allele segregation with other cancers (non-breast, non-ovarian and non-colon) was performed. In other cancers diagnosed under age 50, alleles in 13 out of 17 individuals co-segregated with breast cancer. In 44 other cancer cases diagnosed at any age, alleles in 31 individuals co-segregated with breast malignancy compared with those in 13 which did not.

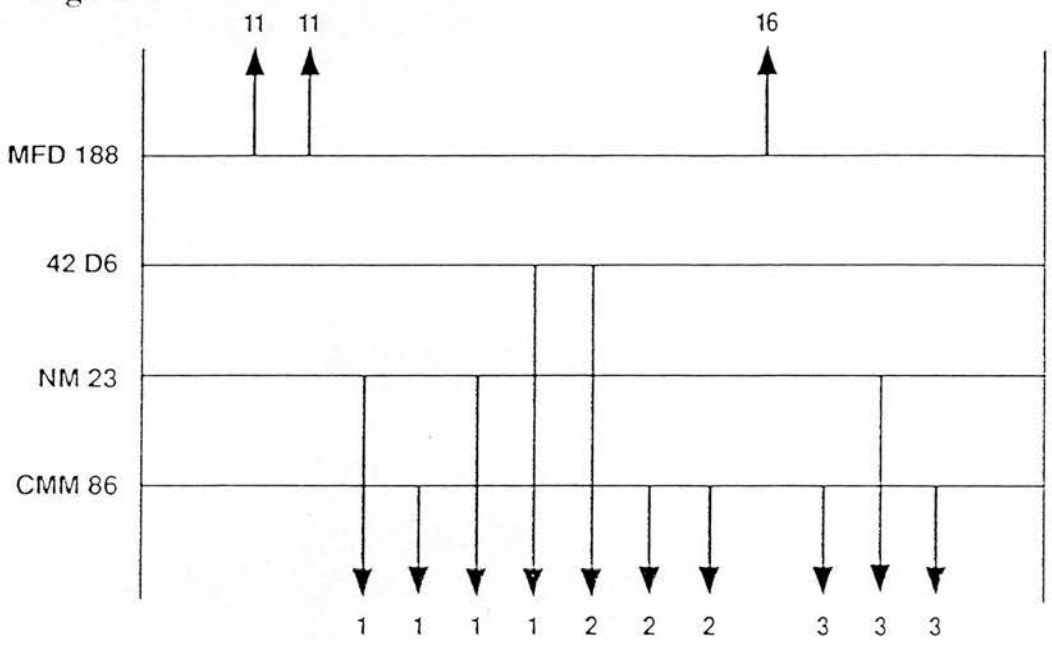
Meiotic recombination of marker alleles occurs with a frequency related to (though in non-linear fashion) the physical distance between markers. We observed recombination between markers in some Edinburgh pedigrees in which disease and 17q markers appear to be linked. At the point of recombination it was possible to identify alleles of markers which failed to segregate with the disease and appeared to be abandoned by the disease phenotype in subsequent generations. If it is also assumed that there is a low frequency of double recombination between markers and a negligible chance of sporadic breast cancer cases in the pedigree, then the chromosomal arm between and beyond these abandoned marker alleles can be excluded as a likely site for the breast cancer gene. This allows a recombination exclusion map to be produced in which each such recombination results in the exclusion of part of chromosome 17q as a candidate site. This is illustrated in Figure 5 by arrows placed on a chromosome 17q frame. Each arrow denotes a single recombination event which

excludes the region covered by the arrow. Only families which appear likely to be linked to a 17q gene are included in this map. Ideotypes of the 5 Edinburgh pedigrees with these critical recombinants are illustrated in Figures 6a and 6b. Haplotypes of all other Edinburgh pedigrees at loci 42D6 and MFD188 are illustrated in Figures 6c, 6d and 6e. Diagnoses of all cancers, together with age at diagnosis are recorded in Table XI with individuals identified in relation to identification numbers shown in figures 6a - 6e.

**Figure 5**

Recombination exclusion map of chromosome 17q. Each arrow denotes a presumed gene carrier in which a meiotic recombination in a marker allele causes failure of the allele to segregate with breast cancer. The arrow covers the part of chromosome 17q which is excluded as a site of the gene. Pedigrees which contain these critical recombinants are identified by number at the arrow heads.

Figure 5



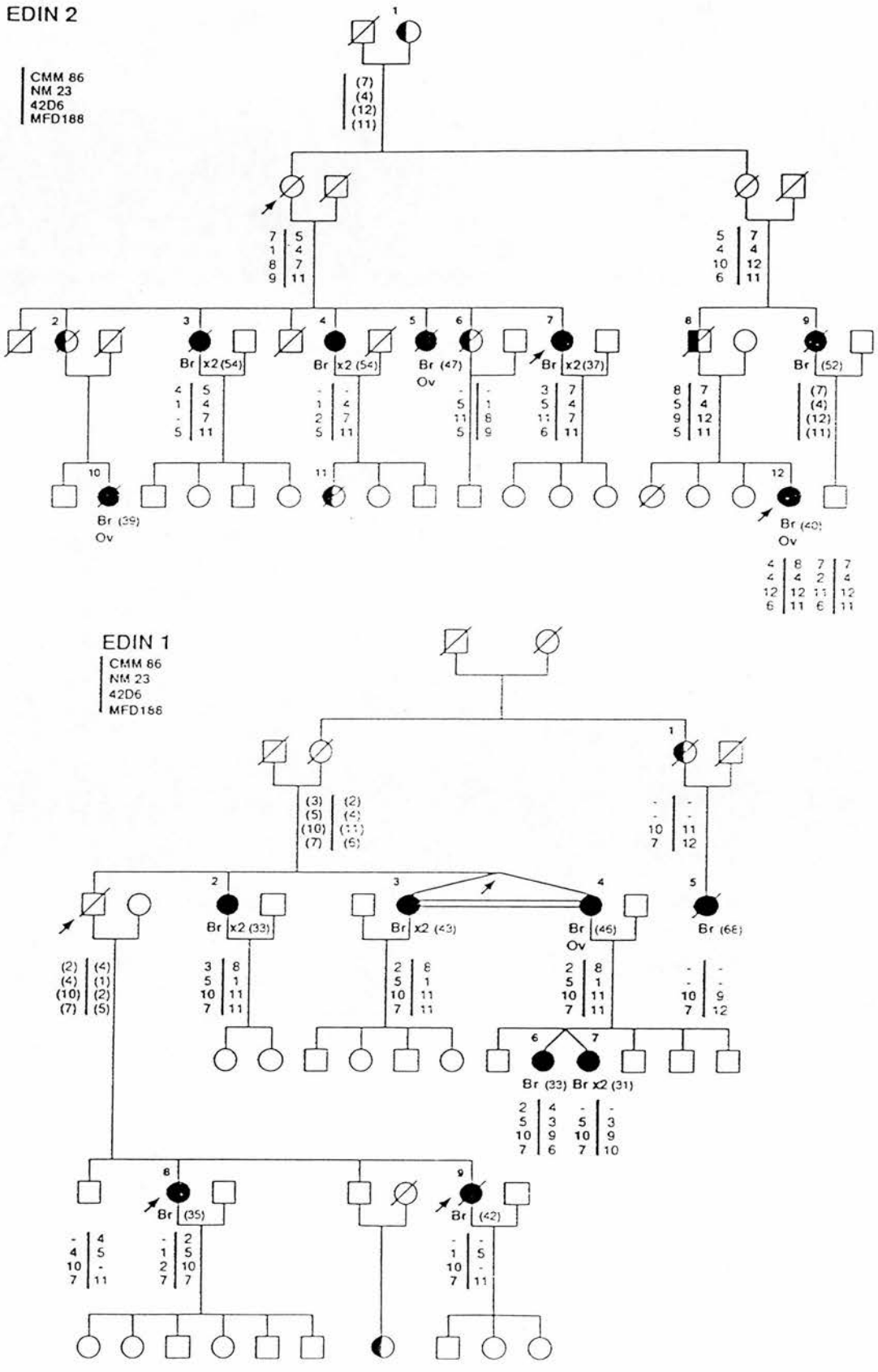
**Figures 6a - 6e**

Fifteen Edinburgh pedigrees with identification numbers of individuals with cancer recorded in detail in Table XI. Haplotypes at 17q markers illustrated. Linked haplotypes are typed in bold.

*Figures 6a, 6b :* Ideotypes of 5 Edinburgh pedigrees illustrating critical recombinants which are arrowed. Position of critical recombinants which may exist in pedigree EDIN 11 impossible to localise.

*Figures 6c, 6d, 6e :* Ideotypes of remaining 10 Edinburgh pedigrees illustrating haplotypes at loci 42D6 and MFD188.

Figure 6a


EDIN 2
CMM 86
NM 23
42D6
MFD188
Br x2 (54)
Br x2 (54)
Br (47)
Ov
Br x2 (37)
Br (52)
Br (39)
Ov
Br (40)
Ov
EDIN 1
CMM 86
NM 23
42D6
MFD188
Br x2 (33)
Br x2 (43)
Br (46)
Ov
Br (68)
Br (33) Br x2 (31)
Br (35)
Br (42)

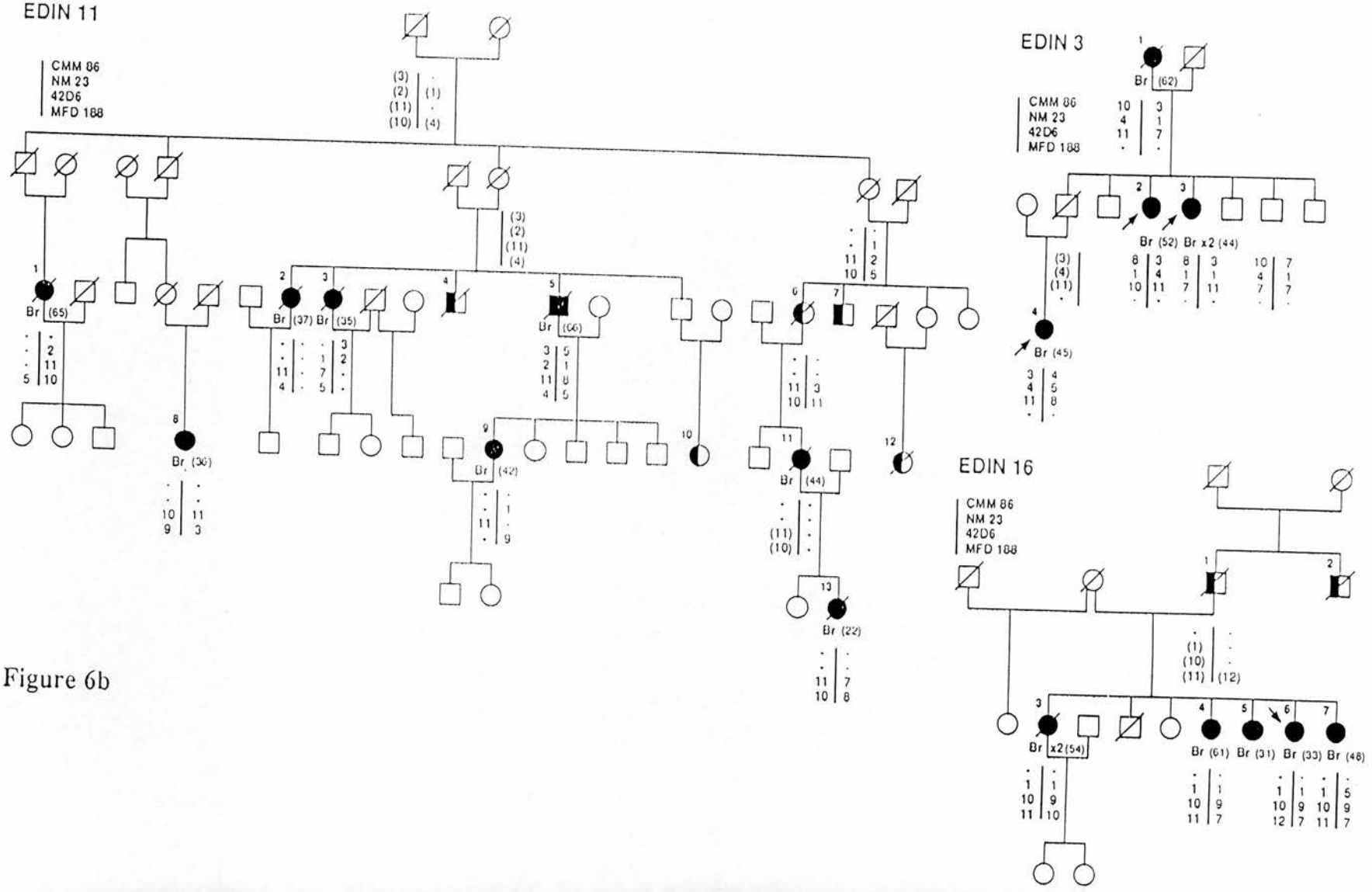

EDIN 11
CMM 86
NM 23
42D6
MFD 188
EDIN 3
CMM 86
NM 23
42D6
MFD 188
EDIN 16
CMM 86
NM 23
42D6
MFD 188

Figure 6b

Figure 6c

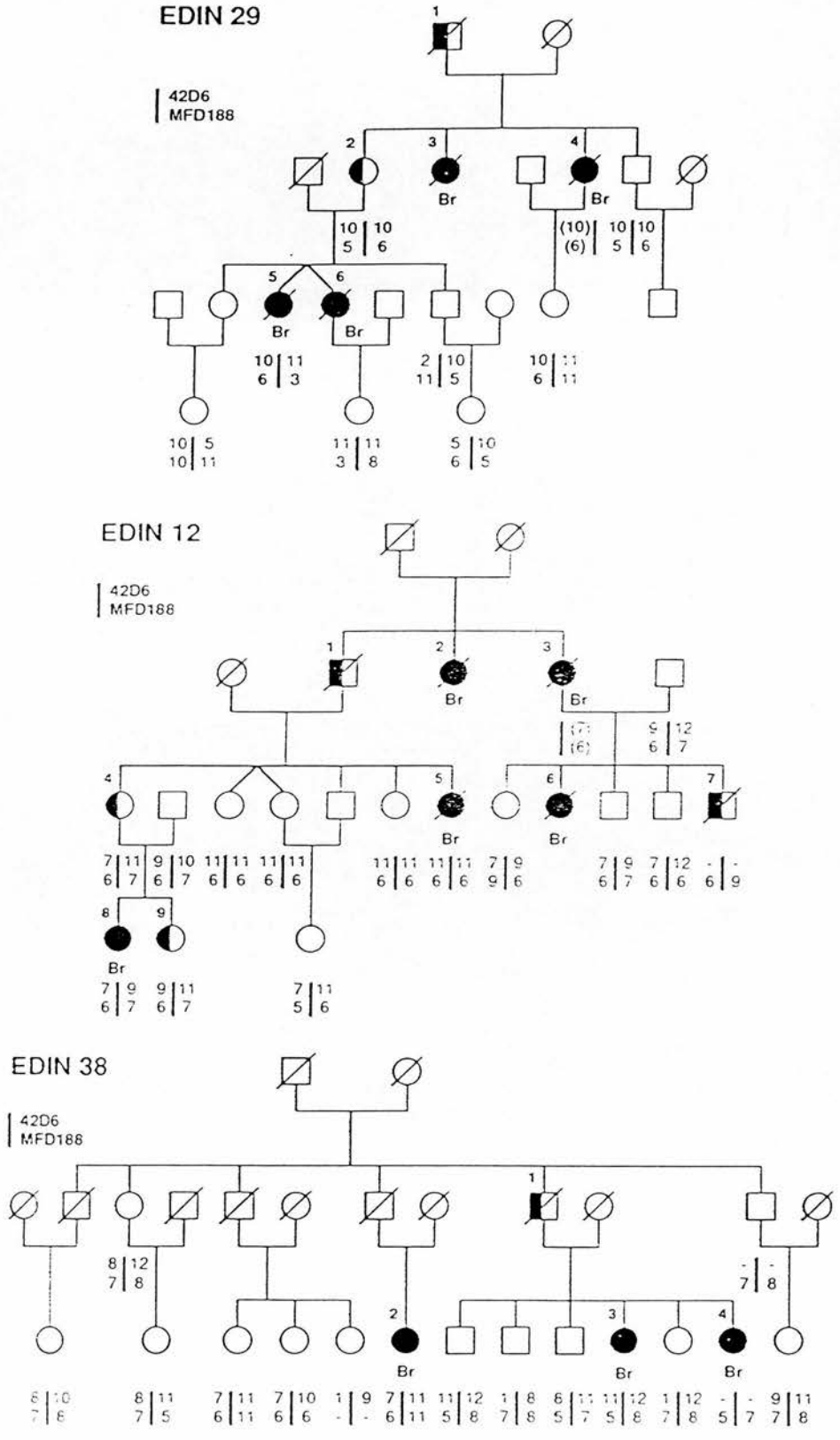
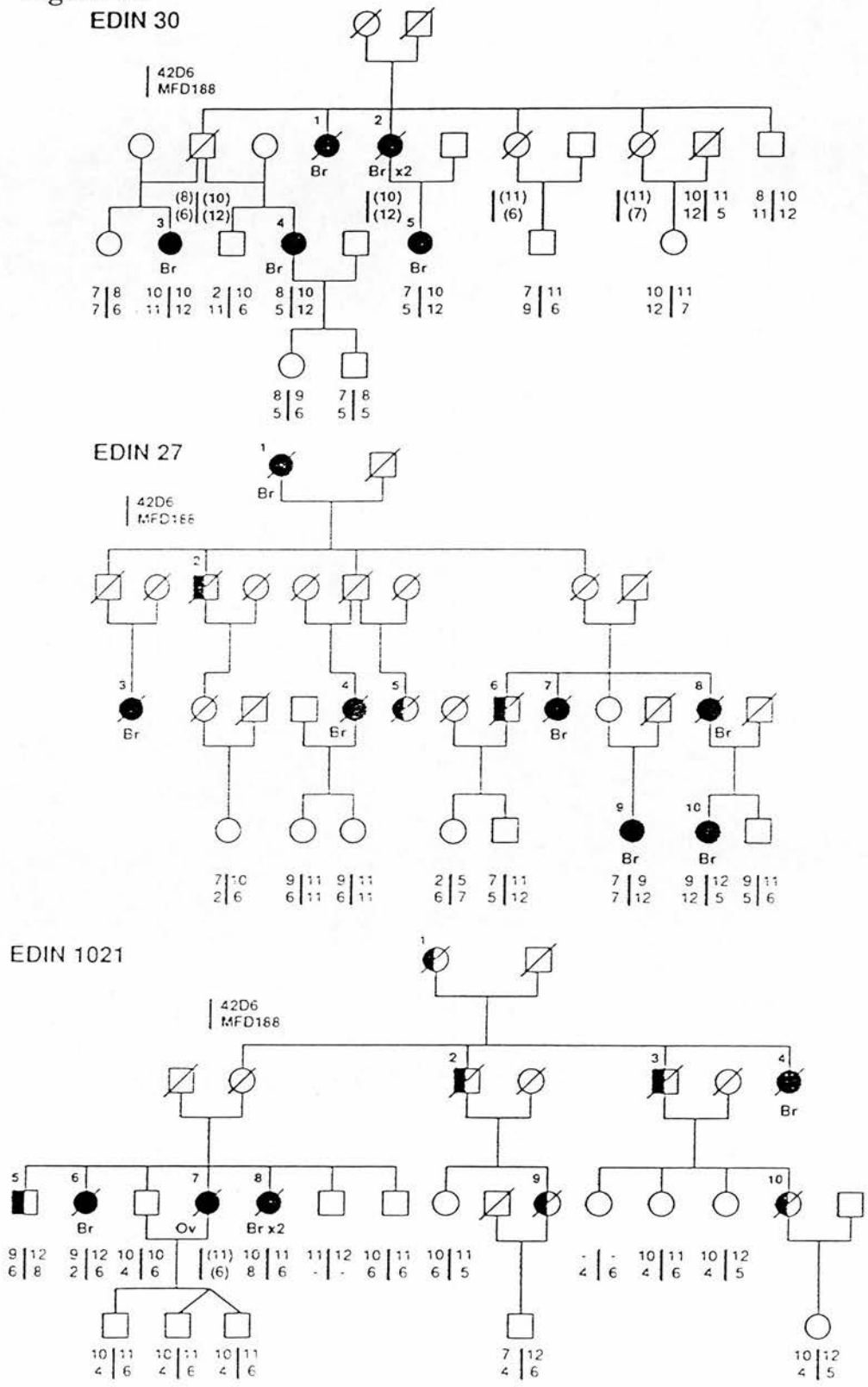


Figure 6d


EDIN 30
42D6
MFD188
Br
Br x2
Br
Br
Br
EDIN 27
42D6
MFD188
Br
Br
Br
Br
Br
Br
EDIN 1021
42D6
MFD188
Br
Br
Ov
Br x2
Br

## Figure 6e

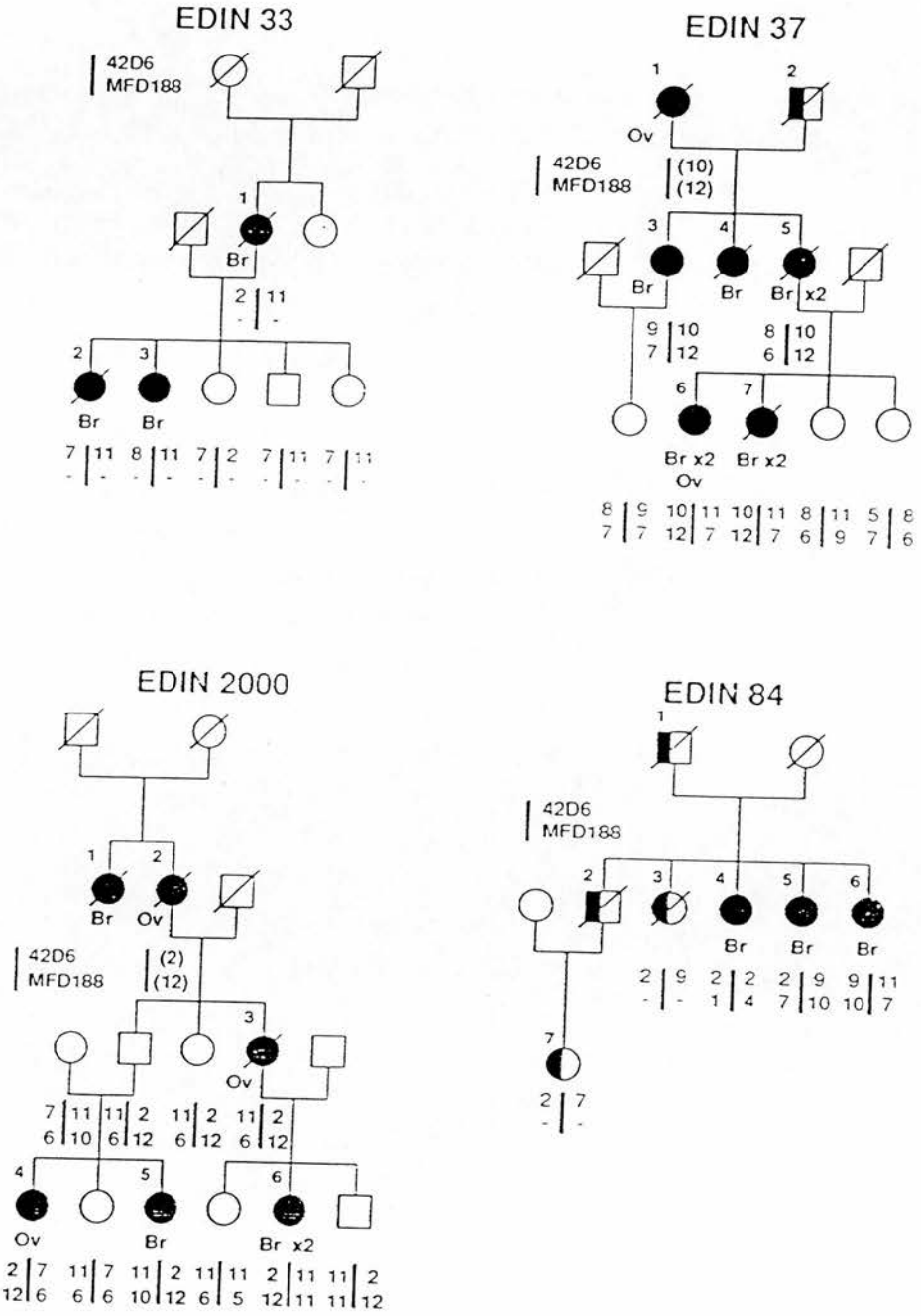

EDIN 33
42D6
MFD188
1
Br
2 11
- -
2
Br
3
Br
7 11 8 11 7 2 7 11 7 11
- - - - - - - - -
EDIN 37
1
Ov
2
42D6
MFD188
(10)
(12)
3
Br
4
Br
5
Br x2
9 10
7 12
8 10
6 12
6
Br x2
Ov
7
Br x2
8 9 10 11 10 11 8 11 5 8
7 7 12 7 12 7 6 9 7 6
EDIN 2000
1
Br
2
Ov
42D6
MFD188
(2)
(12)
3
Ov
7 11 11 2 11 2 11 2
6 10 6 12 6 12 6 12
4
Ov
5
Br
6
Br x2
2 7 11 7 11 2 11 11 2 11 11 2
12 6 6 6 10 12 6 5 12 11 11 12
EDIN 84
1
42D6
MFD188
2
3
4
Br
5
Br
6
Br
2 9 2 2 2 9 9 11
- - 1 4 7 10 10 7
7
2 7
- -

**Table XI**

All diagnoses of cancer with age of diagnosis in 15 Edinburgh pedigrees. Ranking by family and individual identifier as illustrated in Figures 6a - 6e.

# Table XI

| Family | Identifier | Cancer | Age |
|---|---|---|---|
| 1 | 1 | Gastric | 65 |
| 1 | 2 | Breast x2 | 33 |
| 1 | 3 | Breast x2 | 43 |
| 1 | 4 | Breast x2 | 46 |
| | | Ovarian | 67 |
| 1 | 5 | Breast | 68 |
| | | Stomach | 57 |
| 1 | 6 | Breast | 33 |
| 1 | 7 | FAP | 33 |
| | | Breast x2 | 31 |
| 1 | 8 | Breast | 35 |
| 1 | 9 | Breast | 42 |
| 1 | 10 | Cervix | 25 |
| 2 | 1 | Oesophagus | 54 |
| 2 | 2 | Colon | 64 |
| 2 | 3 | Breast x2 | 54 |
| 2 | 4 | Breast x2 | 54 |
| 2 | 5 | Breast | 47 |
| | | Ovarian | 51 |
| 2 | 6 | CNS Tumour | 66 |
| 2 | 7 | Breast x2 | 37 |
| | | Lung | 63 |
| 2 | 8 | Colon | 75 |
| 2 | 9 | Breast | 52 |
| 2 | 10 | Breast | 39 |
| | | Ovarian | 40 |
| 2 | 11 | Squam. Anus | 37 |
| 2 | 12 | Breast | 40 |
| | | Ovarian | 45 |
| 3 | 1 | Breast | 62 |
| 3 | 2 | Breast | 52 |
| 3 | 3 | Breast x2 | 44 |
| 3 | 4 | Breast | 45 |
| 11 | 1 | Breast | 65 |
| 11 | 2 | Breast | 37 |
| 11 | 3 | Breast | 35 |
| 11 | 4 | Prostate | 74 |
| 11 | 5 | Breast | 66 |
| 11 | 6 | Renal | 54 |
| 11 | 7 | Myxoma | 68 |
| 11 | 8 | Breast | 36 |
| 11 | 9 | Breast | 42 |
| 11 | 10 | Fibrosarcoma | 25 |
| 11 | 11 | Breast | 44 |
| 11 | 12 | Leukaemia | 47 |
| 11 | 13 | Breast | 22 |
| 12 | 1 | Lung | 65 |
| 12 | 2 | Breast | 77 |
| 12 | 3 | Breast | 48 |
| 12 | 4 | Basal Cell Ca | 49 |
| 12 | 5 | Breast | 52 |
| 12 | 6 | Breast | 30 |
| 12 | 7 | PNS Tumour | 25 |
| 12 | 8 | Breast | 37 |
| 12 | 9 | Cervix | 38 |
| 16 | 1 | Sarcoma | 39 |
| 16 | 2 | Prostate | 80 |
| 16 | 3 | Breast x2 | 54 |
| 16 | 4 | Breast | 61 |
| 16 | 5 | Breast | 31 |
| 16 | 6 | Breast | 33 |
| | | Lung | 52 |
| 16 | 7 | Breast | 48 |
| 27 | 1 | Breast | 68 |
| 27 | 2 | Lung | 70 |
| 27 | 3 | Breast | 46 |
| | | Lung, Colon | 51 |
| 27 | 4 | Breast | 73 |
| 27 | 5 | Uterus | 65 |
| 27 | 6 | Lung | 79 |
| 27 | | Leukaemia | 79 |
| 27 | 7 | Breast | 71 |
| 27 | 8 | Breast | 43 |
| 27 | 9 | Breast | 58 |
| 27 | 10 | Breast | 43 |
| 29 | 1 | Prostate | 60 |
| 29 | 2 | Colon | 63 |
| | | Squam. Skin | 75 |
| 29 | 3 | Breast | 67 |
| 29 | 4 | Breast | 43 |
| 29 | 5 | Breast | 48 |
| 29 | 6 | Breast | 44 |
| 30 | 1 | Breast | 72 |
| 30 | 2 | Breast x2 | 62 |
| 30 | 3 | Breast | 53 |
| 30 | 4 | Breast | 60 |
| 30 | 5 | Breast | 43 |
| 33 | 1 | Breast | 53 |
| 33 | 2 | Breast | 42 |
| 33 | 3 | Breast | 42 |
| 37 | 1 | Ovarian | 63 |
| 37 | 2 | Lung | 66 |
| 37 | 3 | Breast | 34 |
| 37 | 4 | Breast | 38 |
| 37 | 5 | Breast x2 | 41 |
| 37 | 6 | Breast x2 | 35 |
| | | Ovarian | 46 |
| 37 | 7 | Breast x2 | 33 |
| 38 | 1 | Lung | 70 |
| 38 | 2 | Breast | 48 |
| 38 | 3 | Breast | 44 |
| 38 | 4 | Breast | 42 |
| 84 | 1 | Colon | 67 |
| 84 | 2 | Colon | 69 |
| 84 | 3 | Lymphoma | 69 |
| 84 | 4 | Breast | 71 |
| 84 | 5 | Breast | 52 |
| 84 | 6 | Breast | 59 |
| 84 | 7 | Colon | 49 |
| 1021 | 1 | Sarcoma | 52 |
| 1021 | 2 | Colon | 42 |
| 1021 | 3 | Bladder | 69 |
| 1021 | 4 | Breast | 67 |
| 1021 | 5 | Bladder | 81 |
| 1021 | 6 | Breast | 65 |
| 1021 | 7 | Ovarian | 60 |
| | | Colon | 60 |
| 1021 | 8 | Breast x2 | 47 |
| 1021 | 9 | Lung | 64 |
| 1021 | 10 | Lung | 68 |
| 2000 | 1 | Breast | ?? |
| 2000 | 2 | Ovarian | 60 |
| 2000 | 3 | Ovarian | 54 |
| 2000 | 4 | Ovarian | 40 |
| 2000 | 5 | Breast | 36 |
| 2000 | 6 | Breast x2 | 48 |

**ii) Identification of probable 17q breast cancer gene carriers :**

The probability that any family member has breast cancer as a result of inheritance of a gene on 17q is classically determined through the expression of final, or posterior, probability via initial calculation of prior and conditional probabilities in accordance with Bayes' theorem (Emery, 1986). Linkage analysis provides the conditional probability data. The prior probability that breast-ovarian cancer pedigrees are linked to 17q is conservatively estimated as 75%, compared with 50% for breast cancer pedigrees (Easton *et al,,* 1993). Thus the posterior probability of linkage to 17q can be calculated for each of 15 breast cancer pedigrees according to the equation :

Posterior probability in favour of linkage =

$$\frac{[\text{Prior probability in favour} \times \text{Conditional probability in favour}]}{\text{Prior probability in favour} \times \text{Conditional probability in favour} + \text{Prior probability against} \times \text{Conditional probability against}}$$

Figure 7 illustrates maximal posterior probability of linkage to the 17q breast cancer gene at either 42D6 of MFD188 for each of 15 breast cancer pedigrees. 10 families were considered to be "site-specific" cancer families (no cases of ovarian cancer) and 5 were "breast-ovarian" cancer families (at least one case of ovarian cancer). In a single hypothetical pedigree, a posterior probability of linkage of 75% indicates that the probability that a mutant susceptibility gene close to markers analysed has caused disease in the family is three times more

likely than not. Arbitrarily, therefore, it might be considered that pedigrees with posterior probability of linkage greater than 75% could be viewed as being "probably" linked to the disease-associated gene. 8 out of 15 pedigrees exhibited maximal posterior probabilities of linkage greater than 75% (79.2%, 85.5%, 88.8%, 92.3%, 94.6%, 95.9%, 99.2% and 99.9%). Individuals within these linked families for whom typing at 42D6 and MFD188 suggested they had inherited the marker alleles associated elsewhere in the pedigree with malignancy were identified as probable carriers of the 17q cancer susceptibility gene mutation. Probable non-carriers were identified from among their relatives in a similar fashion. Figure 8 indicates how marker data in a linked pedigree is able to reveal relatives likely to be either carriers or non-carriers of the mutated susceptibility gene. This gene has been assigned the nomenclature BRCA I ( BReast CAncer I ) by the Human Genome Mapping Project.

**Figure 7**

Posterior probability of linkage (%) in Edinburgh pedigrees identified by number and calculated on the basis of prior and conditional odds from 17q marker data. Posterior odds illustrated are derived from whichever single polymorphism (either 42D6 or MFD188) gave the highest LOD score

**Figure 7**

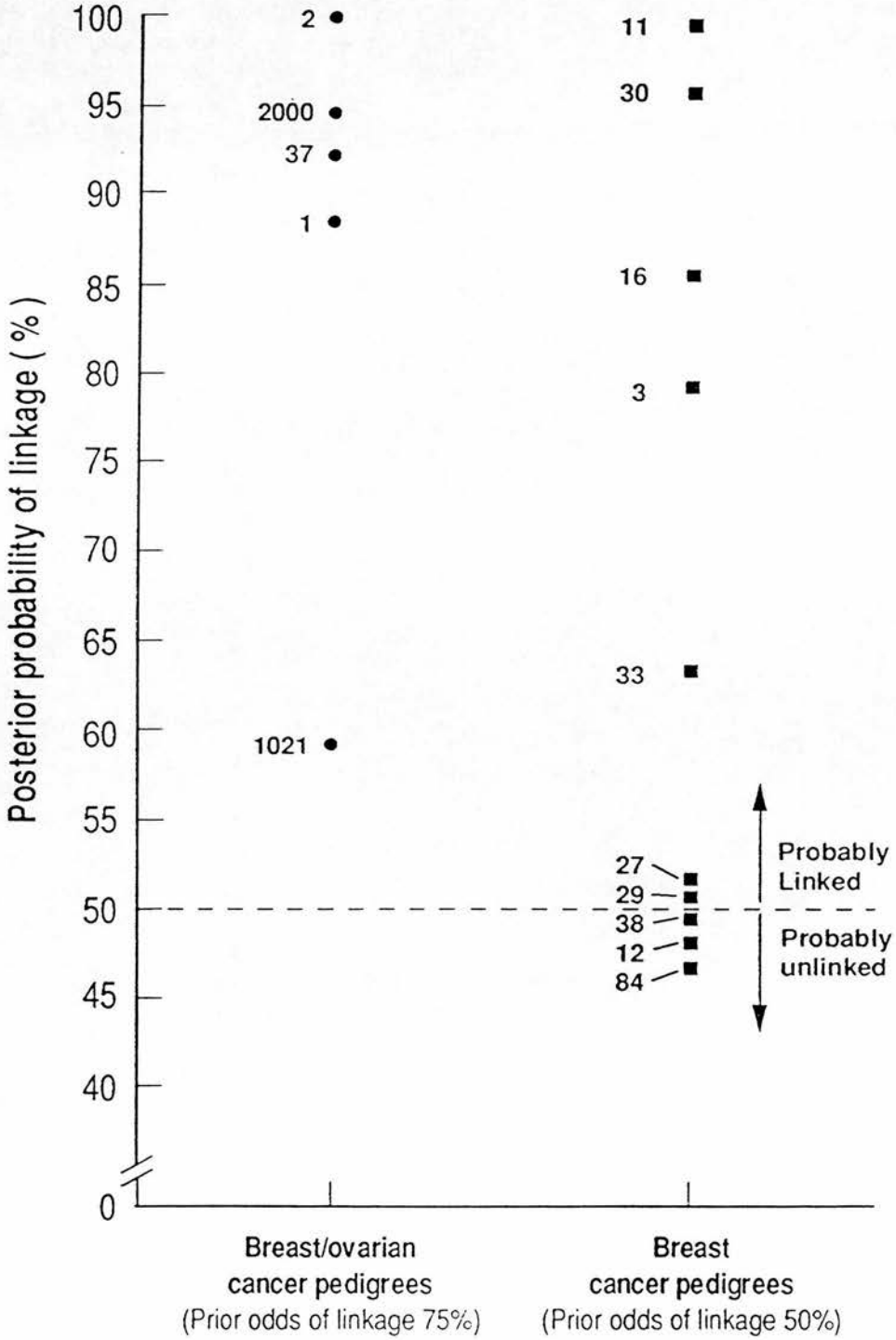
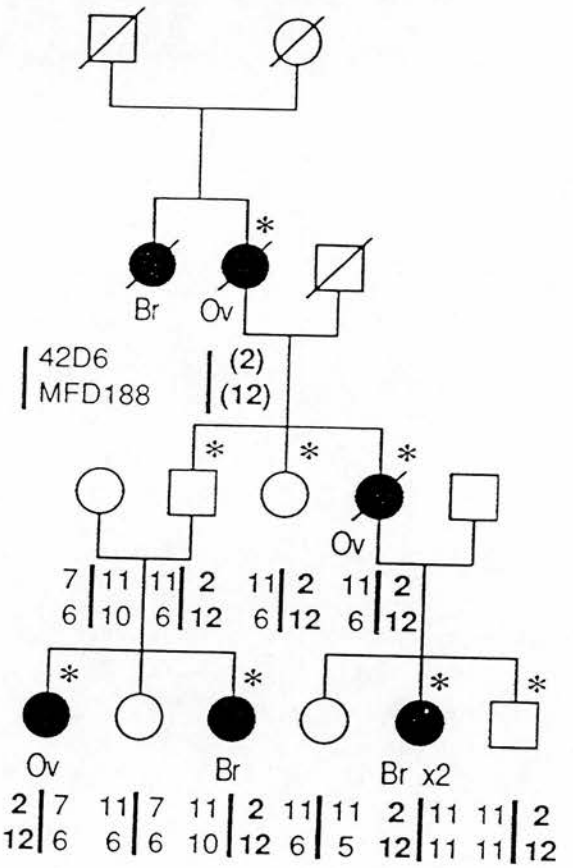

Posterior probability of linkage ( % )
100
95
90
85
80
75
70
65
60
55
50
45
40
0
2
2000
37
1
1021
11
30
16
3
33
27
29
38
12
84
Probably Linked
Probably unlinked
Breast/ovarian cancer pedigrees (Prior odds of linkage 75%)
Breast cancer pedigrees (Prior odds of linkage 50%)

**Figure 8**

Edinburgh pedigree EDIN 2000 with probability of linkage to either 42D6 or MFD188 of over 90% illustrating probable BRCA1 carriers.

**Figure 8**




* Probable carrier of BRCA1 gene mutation

### iii) Penetrance, age-incidence and survival in 17q breast cancer gene carriers.

A total of 102 female relatives from the 8 linked pedigrees were typed in sufficient detail to determine their probable carrier status. 61 women were classified as probable BRCA I mutation carriers; 22 of these had been diagnosed with unilateral breast cancer and 13 with bilateral disease. Date of breast cancer diagnosis was recorded as date of histological confirmation. Mean age of first breast cancer in this group was 43.4 years (standard error 1.7 years). Median year of first diagnosis was 1974 (range 1942 - 1992). 41 women were probable non-carriers of BRCA I mutation (Figure 9).

Genetic penetrance of the gene in BRCA1-linked pedigrees was determined by analysis of the proportion of affected and unaffected BRCA I mutation carriers at given age-intervals. The resultant BRCA I mutation penetrance function is shown in graphic form in Figure 10. For comparison, the segregation analysis penetrance function of a gene responsible for the totality of heritable breast cancer, as calculated by Iselius *et al* (1991) is also illustrated. The proportion of BRCA I mutation carriers diagnosed with breast cancer was 15% by age 35, 46% by age 45 and 88% by age 65.

The cumulative age-incidence curve for BRCA I mutation carriers is shown graphically in Figure 11 together with a Scottish population curve for comparison from the period 1971 - 1973 (Cancer Registration and Survival Statistics Scotland, 1981). Over 90% of

breast cancer diagnoses and deaths within the 61 BRCA I mutation carriers occurred in Scotland. 60% of the total first diagnosis of breast cancer occurred by age 45 and, in this cohort, 100% by age 65.

**Figure 9**

Venn diagram illustrating number of individuals with breast or ovarian cancer and their genotypes in female relatives from 8 BRCA1-linked pedigrees.

Figure 9

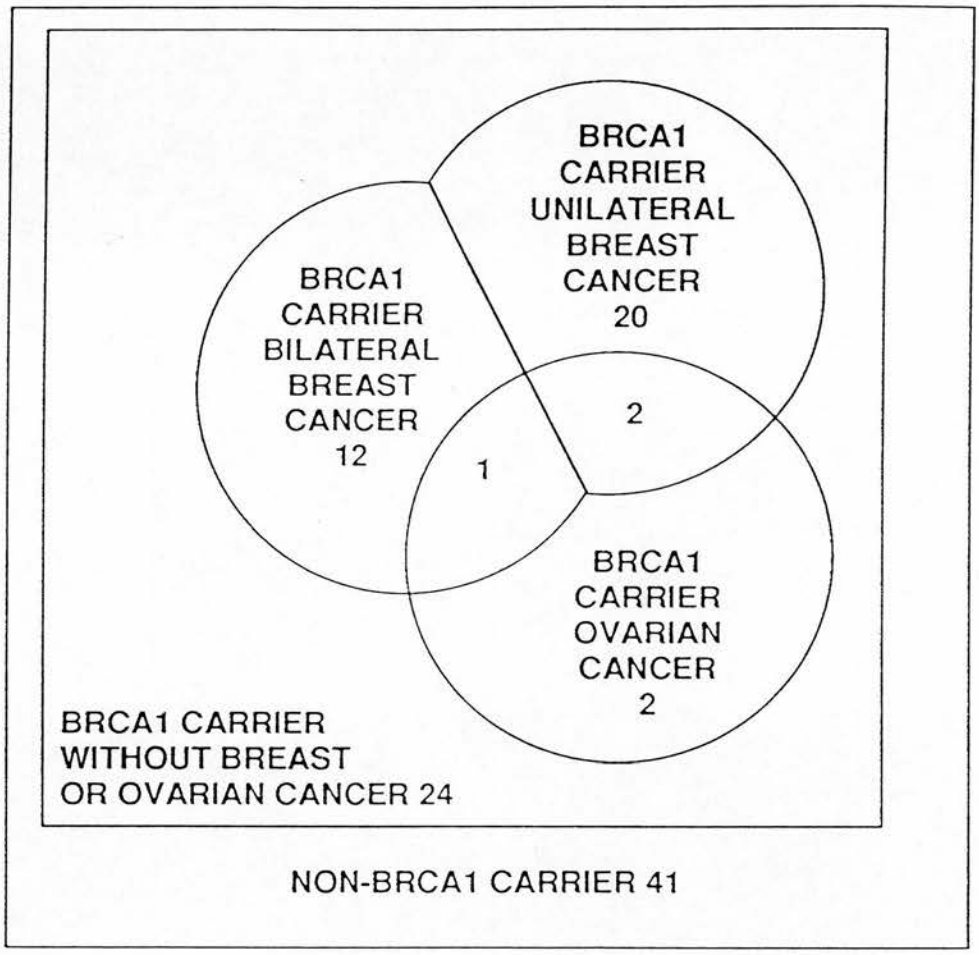

BRCA1
CARRIER
UNILATERAL
BREAST
CANCER
20
BRCA1
CARRIER
BILATERAL
BREAST
CANCER
12
2
1
BRCA1
CARRIER
OVARIAN
CANCER
2
BRCA1 CARRIER
WITHOUT BREAST
OR OVARIAN CANCER 24
NON-BRCA1 CARRIER 41

**Figure 10**

Cumulative age-specific breast cancer penetrance in BRCA1 mutation carriers and, for comparison, penetrance calculated in carriers of a hypothetical "totality of breast cancer gene" (Iselius *et al.*, 1991).

**Figure 10**

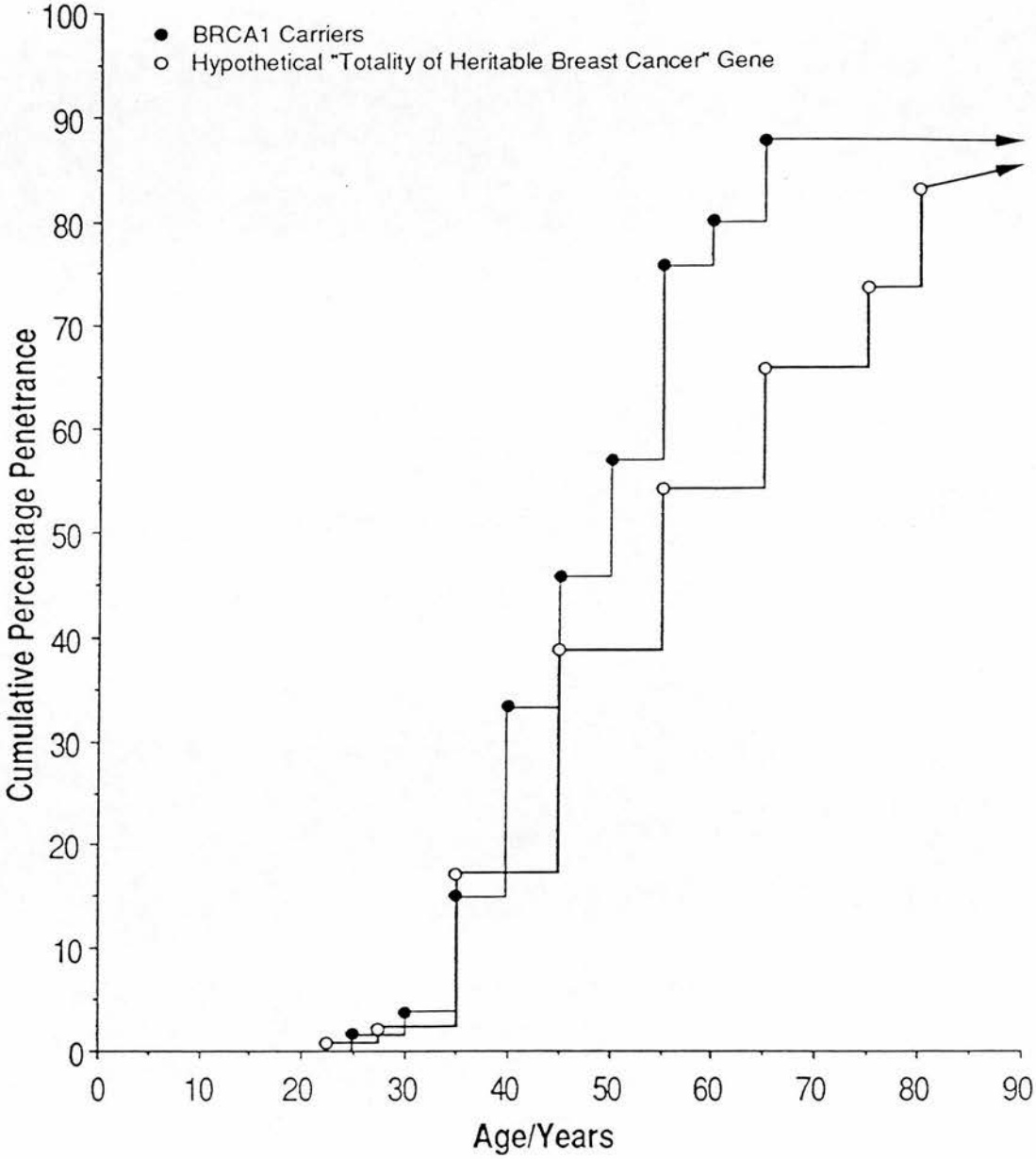
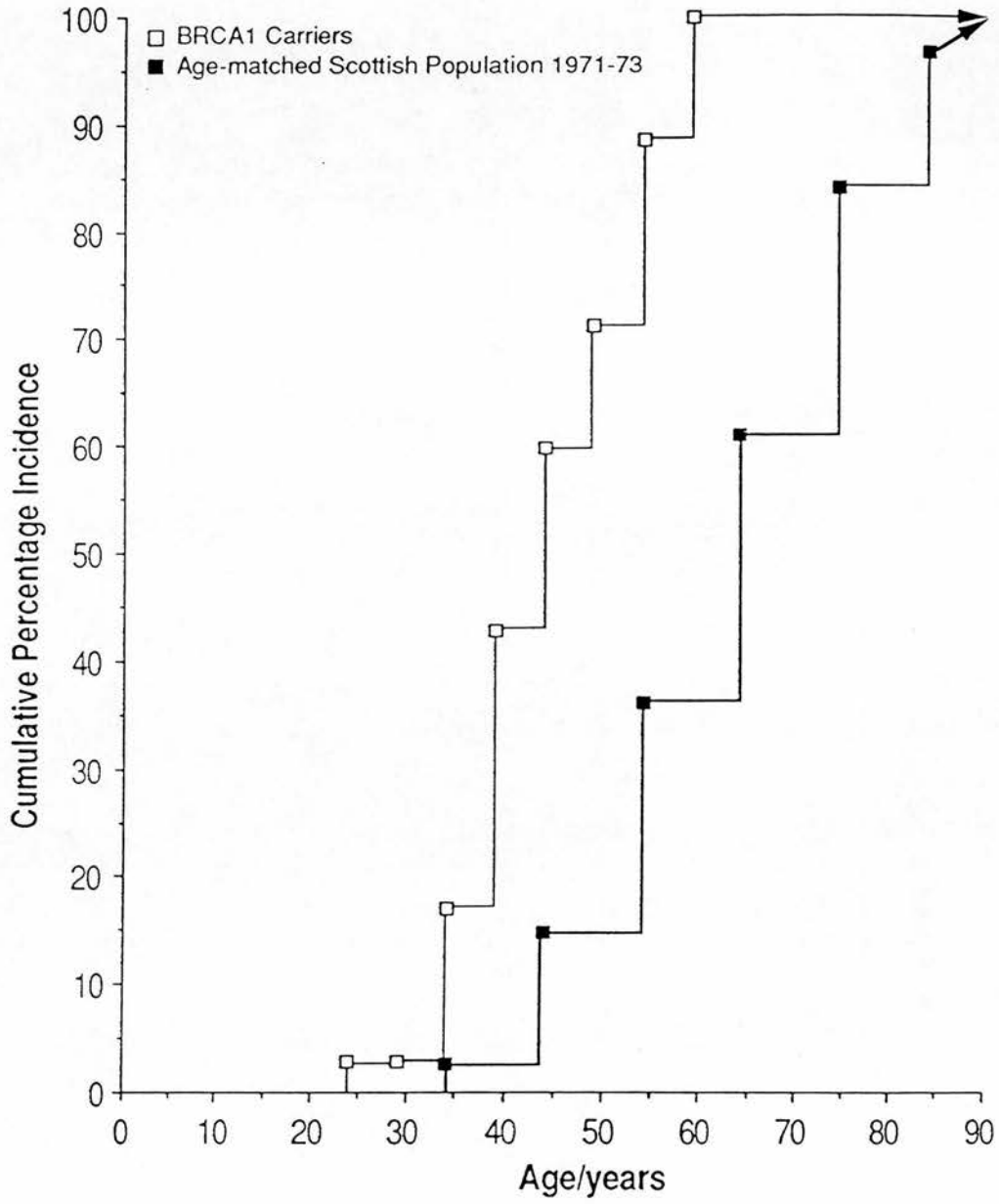

BRCA1 Carriers
Hypothetical "Totality of Heritable Breast Cancer" Gene
Cumulative Percentage Penetrance
Age/Years
0
10
20
30
40
50
60
70
80
90
100

**Figure 11**

Cumulative breast cancer age-incidence in BRCA1 mutation carriers and, for comparison, in the Scottish population (Cancer Registration and Survival Statistics Scotland 1963 - 77. 1981).

**Figure 11**

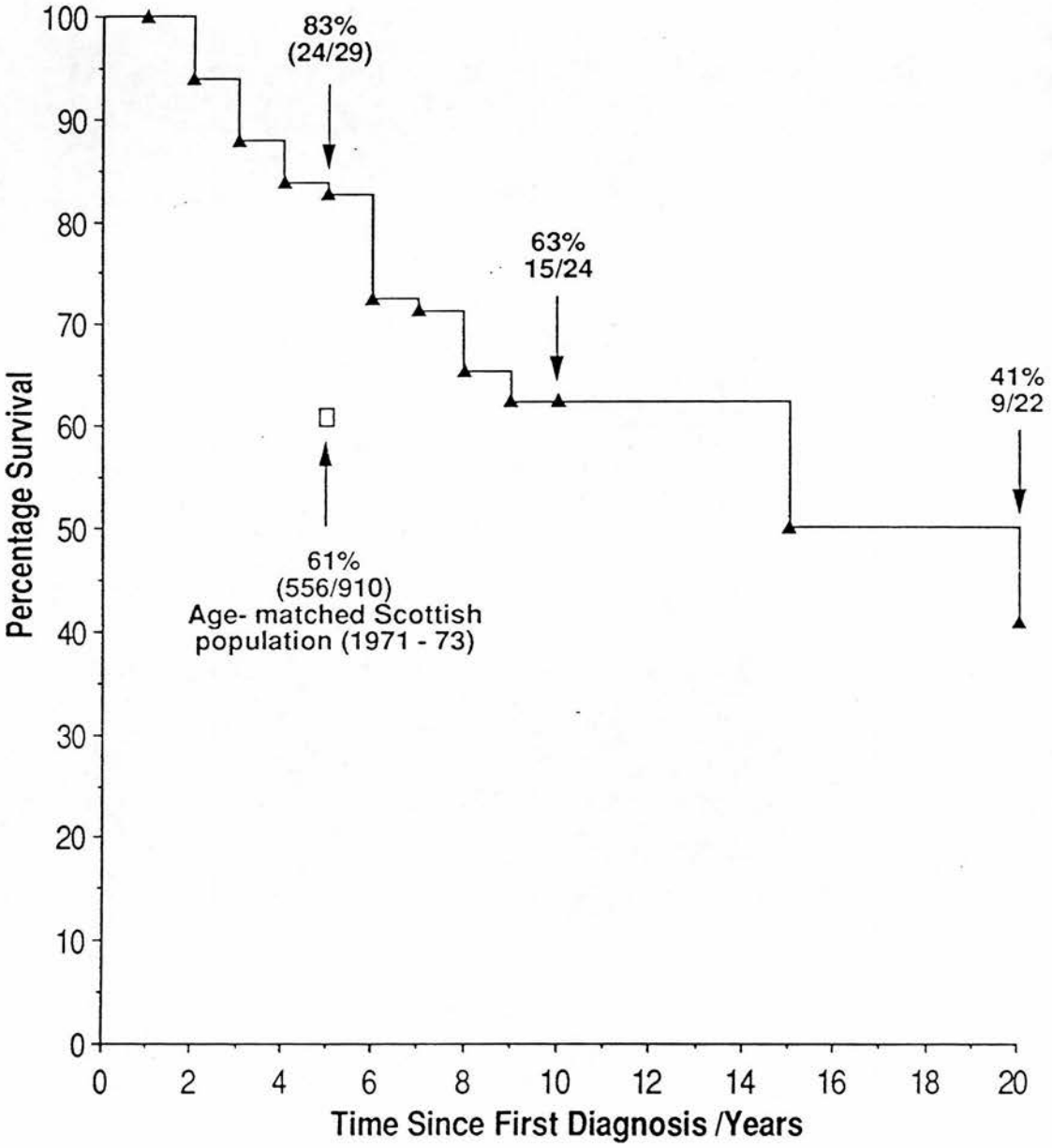

BRCA1 Carriers
Age-matched Scottish Population 1971-73
Cumulative Percentage Incidence
Age/years
0
10
20
30
40
50
60
70
80
90
100

The survival curve for BRCA I mutation carriers with breast cancer is illustrated in Figure 12. 5, 10 and 20 year survival rates were 83% (24/29 individuals), 63% (15/24) and 41% (9/22) respectively. Population tables revealed that 5 year survival in an age-matched group of 910 Scottish patients with breast cancer diagnosed during the period 1971 - 1973 was 61.1% (Cancer Registration and Survival Statistics Scotland, 1981). This figure is significantly different from the 83% 5 year survival rate in BRCA I mutation carriers ($X^2 = 4.70$, $p < 0.05$). Survival data were also examined in 24 individuals with breast cancer from 7 pedigrees not obviously linked to 17q12-21 (posterior odds of linkage ranged from 41.4% to 63.5%). In this group 5 year survival was 59% (10 out of 17 individuals). In contrast to the findings in carriers of BRCA I mutation there was no significant difference between this figure and expected survival taken from age-matched Scottish cancer registration data (also 59%).

**Figure 12**

Cumulative overall survival since first diagnosis of breast cancer in BRCA1 mutation carriers (median year of diagnosis 1974). Five year survival in an age-matched Scottish population is also shown (Cancer Registration and Survival Statistics Scotland 1963 - 77. 1981).

**Figure 12**


100
90
80
70
60
50
40
30
20
10
0
Percentage Survival
83%
(24/29)
63%
15/24
41%
9/22
61%
(556/910)
Age- matched Scottish
population (1971 - 73)
0
2
4
6
8
10
12
14
16
18
20
Time Since First Diagnosis /Years

## 3. Structural chromosome analysis.

Peripheral blood lymphocytes were karyotyped in 104 blood relatives in 18 pedigrees which contained at least two individuals with breast cancer. 16 pedigrees had a low frequency of constitutional chromosome rearrangements (Table XIIa). In these families, 7 separate mosaicism involving 6individuals were found. 6 of these involved mosaicism for a 45X cell line. In addition there were 3 constitutional variants. No evidence for co-segregation of the same anomalies in other family members was observed. No individual with mosaicism or variant had obvious developmental defects.

**Table XIIa**

Details of individuals in which 100 cells were examined exhibiting

a) constitutional chromosomal variants and

b) chromosomal mosaicisms in the 14 Edinburgh pedigrees which exhibited rates of variants and mosaicisms similar to that in the general population.

## Table XIIa

| CONSTITUTIONAL VARIANTS | COMMENTS |
|---|---|
| 46XX<br>21ph+ | Unaffected age 41 from a family<br>**not** showing linkage to 17q |
| 46XY<br>22s+ | Unaffected age 73 from a family<br>**not** showing linkage to 17q |
| 46XX<br>21s+ | Unaffected age 49 from<br>unlinked pedigree |

| MOSAICISMS | (Proportion) | COMMENTS |
|---|---|---|
| 45X/<br>46XX | 8/100<br>92/100 | Ca breast age 45, venesection age 47.<br>Pedigree odds of 17q linkage = 79.2% |
| 45X/<br>46XX | 7/100<br>93/100 | Ca breast age 42, venesection age 51<br>from pedigree **not** showing 17q linkage |
| 45X/<br>46XX/<br>47XXX | 4/100<br>95/100<br>1/100 | Unaffected age 56, venesection age 54.<br>Carrier of 17q susceptibility gene :<br>Pedigree odds of linkage = 88.8% |
| 46XX/<br>46XX<br>del(7)(p11.2) | 90/100<br><br>10/100 | Unaffected at age 55, venesection age 55.<br>Non-carrier of 17q susceptibility gene :<br>Pedigree odds of linkage = 95.9% |
| 45X/<br>46XX | 8/100<br>92/100 | Unaffected age 46 from pedigree<br>**not** showing 17q linkage |
| 45X/<br>46XX | 10/100<br>90/100 | Unaffected age 66 from pedigree<br>**not** showing 17q linkage |

Members of one pedigree exhibited a variety of constitutional chromosomal variants, several of which were shown to be present in more than one relative. This complex distribution of cytogenetic anomalies is illustrated in Figure 13.

One further family illustrated as pedigree A in Figure 2 in which a germline point mutation in the p53 gene was segregating failed to fulfil criteria for classification as Li-Fraumeni syndrome (Prosser *et al.*, 1992). On cytogenetic analysis, two members of this kindred had both germline p53 mutation and constitutional chromosome aberrations. These are shown in Figure 14.

In 8 pedigrees an analysis of LOD scores at markers located on chromosome 17q12-21 suggested that posterior odds of linkage to a major breast cancer susceptibility gene on chromosome 17q (BRCA I) were greater than 75% (Figure 7). In seven of these linked pedigrees, individuals had been analysed for constitutional chromosome aberrations. 13 such individuals could be identified as probable carriers of BRCA I gene mutations on the basis of marker data. Constitutional chromosome analysis in these were compared with 12 age-matched non-breast cancer gene carriers from these same 7 pedigrees (mean age in each group 36 years, sex ratios approximately equal). The proportion of cells exhibiting fragile site breaks, chromosome loss and chromosome anomalies in general were significantly less in BRCA I carriers than in non-carriers (Table XIIb). A widespread distribution and frequency of single-cell

breakpoints throughout the genome in both gene carriers and non-carriers is illustrated in Figure 15.

**Figure 13**

An Edinburgh pedigree (EDIN 1021 in Figure 1) illustrating complex karyotypes of individuals.

**Figure 13**

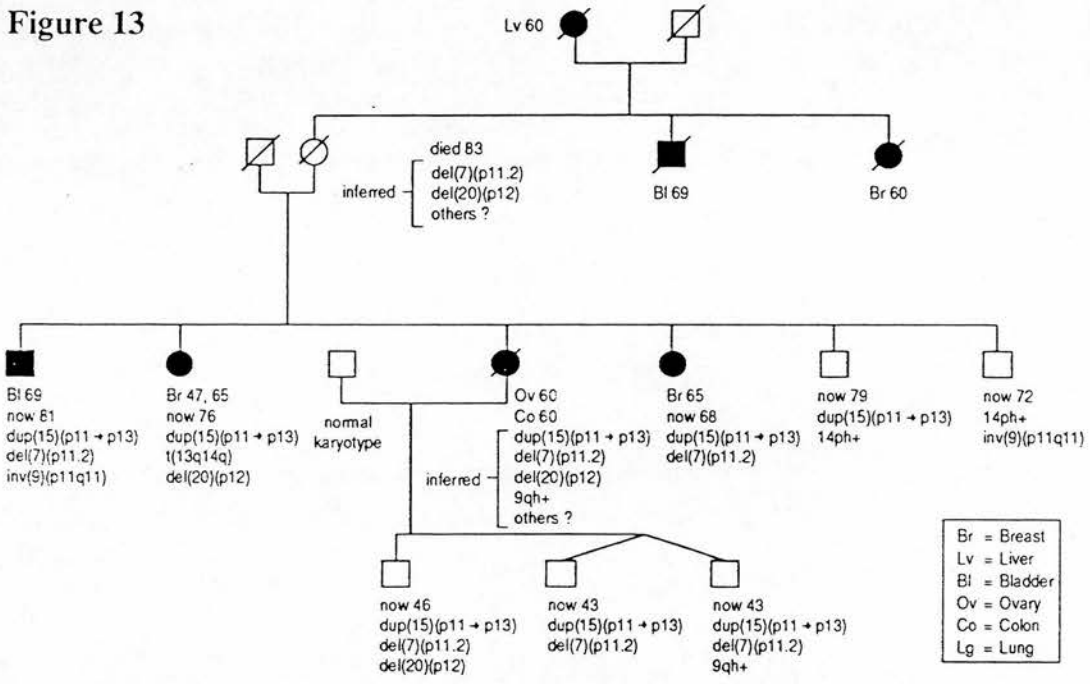

Lv 60
died 83
inferred
del(7)(p11.2)
del(20)(p12)
others ?
Bl 69
Br 60
Bl 69
now 81
dup(15)(p11 → p13)
del(7)(p11.2)
inv(9)(p11q11)
Br 47, 65
now 76
dup(15)(p11 → p13)
t(13q14q)
del(20)(p12)
normal
karyotype
Ov 60
Co 60
inferred
dup(15)(p11 → p13)
del(7)(p11.2)
del(20)(p12)
9qh+
others ?
Br 65
now 68
dup(15)(p11 → p13)
del(7)(p11.2)
now 79
dup(15)(p11 → p13)
14ph+
now 72
14ph+
inv(9)(p11q11)
now 46
dup(15)(p11 → p13)
del(7)(p11.2)
del(20)(p12)
now 43
dup(15)(p11 → p13)
del(7)(p11.2)
now 43
dup(15)(p11 → p13)
del(7)(p11.2)
9qh+
Br = Breast
Lv = Liver
Bl = Bladder
Ov = Ovary
Co = Colon
Lg = Lung

**Figure 14**

An Edinburgh pedigree (Pedigree A in Figure 2) illustrating individuals with germline p53 gene mutations and karyotypic status.

# Figure 14

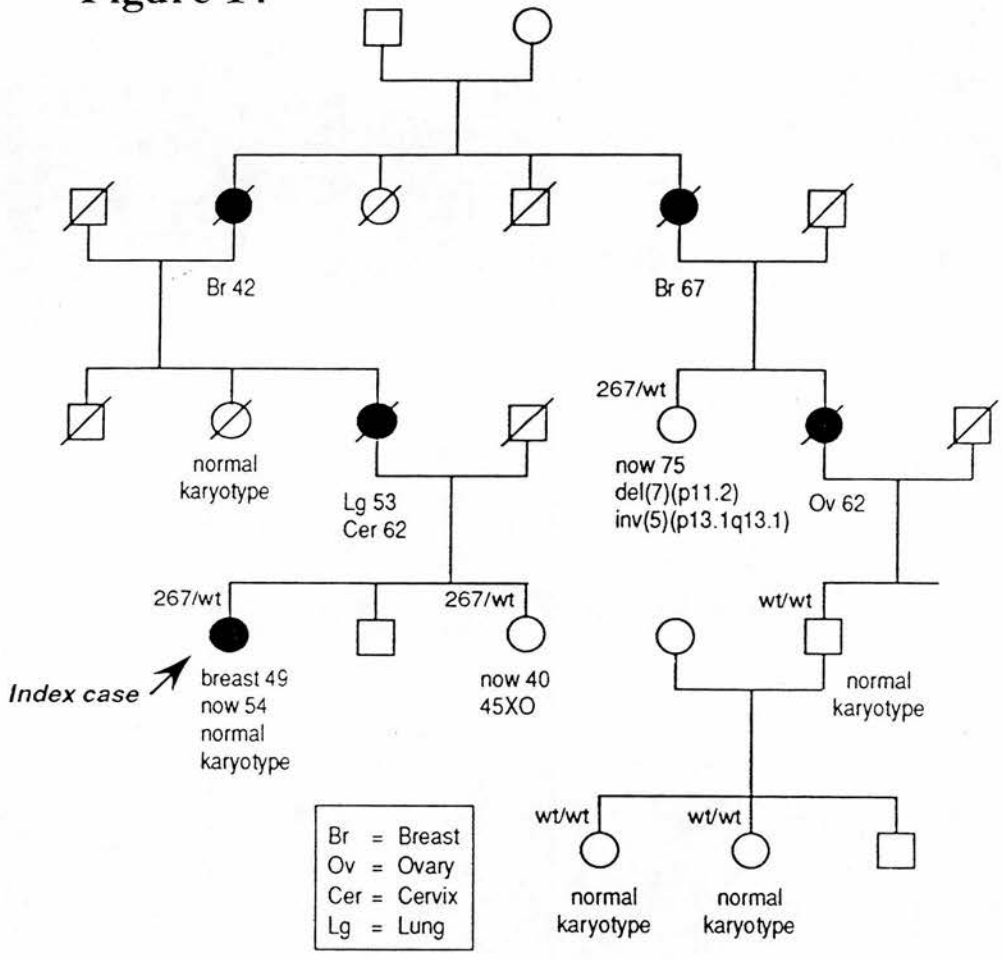

Br 42
Br 67
normal karyotype
Lg 53
Cer 62
267/wt
now 75
del(7)(p11.2)
inv(5)(p13.1q13.1)
Ov 62
267/wt
267/wt
wt/wt
Index case
breast 49
now 54
normal karyotype
now 40
45XO
normal karyotype
Br = Breast
Ov = Ovary
Cer = Cervix
Lg = Lung
wt/wt
wt/wt
normal karyotype
normal karyotype

**Table XIIb**

Actual numbers and percentage of cells with chromosomal anomalies in BRCA1 mutation carriers and non-carriers. Levels of significant difference are also recorded.

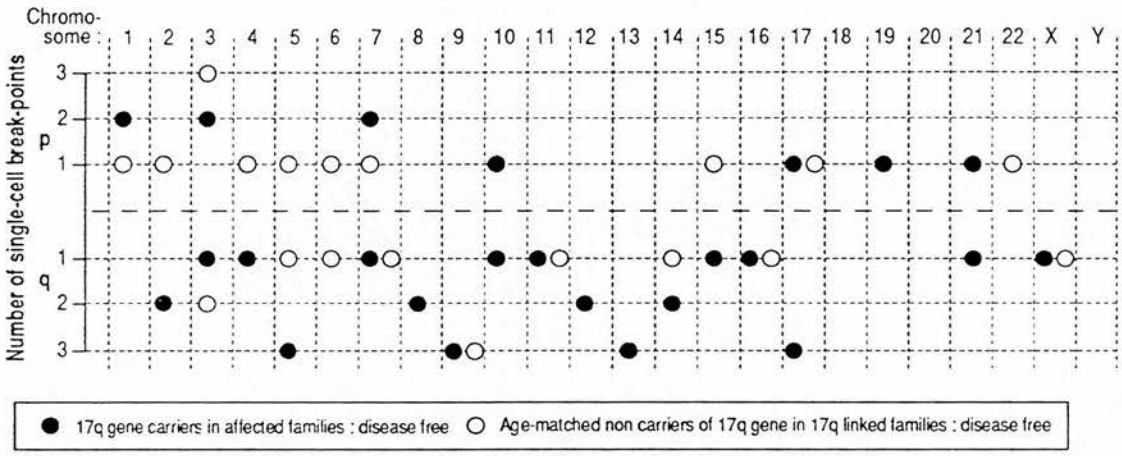
## Table XIIb

| Linkage status of unaffected individuals in BRCA1-linked families | Fragile site breaks (%) | Chromosome loss (%) | Total anomalies (%) |
|---|---|---|---|
| BRCA1 carriers (1030 cells exmined) | 12 (1.17) | 76 (7.38) | 125 (12.14) |
| Non BRCA1 carriers (389 cells exhamined | 11 (2.83) | 48 (12.34) | 75 (19.28) |
| Level of significant difference (chi-square) | 0.05 | 0.01 | 0.001 |

**Figure 15**

Distribution of single cell break-points by chromosome location and number of break-points in both carriers and non-carriers of BRCA1 gene mutation.

**Figure 15**


Chromo-
some :
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
Number of single-cell break-points
p
q
3
2
1
1
2
3
● 17q gene carriers in affected families : disease free
○ Age-matched non carriers of 17q gene in 17q linked families : disease free

# DISCUSSION

## 1. p53 mutation analysis.

## 2. Linkage analysis.

i) Two point LOD series.

ii) Penetrance, age-incidence and survival in 17q breast cancer gene carriers.

## 3. Structural chromosome analysis.

## 1. p53 mutation analysis

Pedigree A in Figure 2 contains 5 close female relatives with breast cancer and other tumours, yet does not fulfil criteria for classification as Li-Fraumeni syndrome (Li *et al*, 1988). Mean age of cancer development in these 5 individuals is 54 years. This compares with a mean age of cancer diagnosis of 25 years in 8 Li-Fraumeni syndrome families with germline p53 gene mutation (Santibanez-Koref *et al.*, 1991b). It can be inferred from DNA analysis that 4 individuals with malignancy in pedigree A inherited mutant p53 at codon 267 (no tissue was available for p53 genotyping from the fifth). This particular germline mutation had not been previously published, although a review of 326 tumour and germline p53 gene mutations by De Fromental and Soussi (1992) lists one small-cell lung cancer cell line mutation reported within this codon (CGG → CCG; Arginine → Proline).

In pedigree A, the p53 gene mutation is found in 37 and 74 year old cancer-free relatives. It is therefore possible that the "mutation" is rather an incidental polymorphism producing a base change without phenotypic effect. Although codon 267 is situated between highly conserved regions IV and V of the gene, the arginine residue which it encodes is evolutionarily conserved throughout species. It is therefore more likely that the substitution of glutamine for arginine has produced a non Li-Fraumeni syndrome spectrum of disease with incomplete lifetime penetrance. Recent reports suggest that screening of early onset breast cancer patients for germline p53 gene mutation

can identify carriers within whose family there is a significant autosomal dominant pattern of breast cancer inheritance but which lacks the typical features of Li-Fraumeni syndrome (Andersen, 1992).

Pedigree B in Figure 2 fulfils the strict criteria for Li-Fraumeni syndrome classification (Li *et al.*, 1988). Furthermore, an identical germline p53 gene mutation in codon 273 (CGT → CAT; Arginine → Histidine) has been reported in a Li-Fraumeni syndrome family from the USA (De Fromental and Soussi, 1992). In evolutionary terms, codon 273 in exon 8 of the gene is located within highly conserved domain V. Codon 273 is the third most frequent site of p53 gene mutations reported world-wide (7.5% of total mutations in the gene, (De Fromental and Soussi, 1992).

Pedigree B includes 2 cases of breast cancer, both occurring at relatively young age. This observation supports that of Santibanez-Koref *et al.* (1991a) in which 4 out of 12 individuals with p53 gene mutations in Li-Fraumeni syndrome families were noted to have early onset breast cancer and suggests that a small but significant proportion of early onset familial breast cancer occurs due to inheritance of p53 gene mutations.

## 2. Linkage analysis

The genetic model for familial breast cancer projected by Iselius *et al* (1991) and used in the pedigree analysis program MENDEL (Lange *et*

*al.*, 1988) was chosen in preference to that estimated in the larger CASH (Cancer and Steroid Hormone) survey (Claus *et al.*, 1991). One reason for this was that the population examined by Iselius was British. It was considered that alternative population studies from the USA may have been less likely to reflect the genetic pool predominant in the British Isles. In addition, the association of cases of ovarian cancer and possibly colonic malignancy and sarcoma found in the segregation analysis study of Iselius *et al.* (1991) seemed to be matched by a similar spectrum of associated malignancy in Edinburgh breast cancer pedigrees. Furthermore, the estimated gene frequency and penetrance function agreed with other recent studies in the UK and other observations in Edinburgh pedigrees which suggested that the proportion of gene-carrying women who actually develop breast cancer in their lifetime is rather less than the 92% predicted by the CASH study.

**i) Two-point LOD series**

*Chromosome 17 short arm*

An earlier two point LOD analysis at YNZ 22.1 in pedigree 1 calculated a maximal LOD of +1.8 assuming zero recombination, providing initial optimism that 17p13 might be the site of a heritable breast cancer gene (MacKay, 1989). When additional relatives of pedigree 1 were screened, however, the maximal LOD was reduced to +1.475. Four further families were screened at this locus (Table III). Taken together there was no evidence of tight linkage to this region,

although a maximal LOD of +0.74 was achieved assuming a recombination fraction of 10%.

YNZ 22.1 is situated at a locus with significant (60%) loss of constitutional heterozygosity in blood-breast tumour pairs (MacKay *et al.*, 1988; Coles *et al.*, 1990). It appears from this analysis, however, that a mutated tumour suppressor gene in this region rarely, if ever, mediates a heritable trait.

*Chromosome 17 long arm;*

The results from markers located on chromosome 17q provide strong evidence for the existence of a heritable breast cancer gene in the region 17q12-21. As expected, large families with many affected individuals such as pedigrees 1, 2 and 11 tend to contribute more to the overall LOD than small ones (Table X). Average age of onset of breast cancer was previously found to be important in determining families likely to be linked to 17q (Hall *et al.*, 1990, 1992). The present study provides support for this, with a few highly positive LOD scores occurring in families where there is a young average age of onset. This observation may be due in part to the nature of the model estimated by segregation analysis studies and used in the pedigree analysis. The model considers breast cancer at young age as less likely to be sporadic, and therefore attributes greater significance to the associated allele data, making LOD scores more highly positive if disease and marker are linked. In families 84 and 1021, average age of onset of breast cancer is 60.7 and 56.0 respectively, and LOD scores from these families do not contribute towards overall positive linkage.

Pedigree 84 also fulfils the criteria for hereditary non-polyposis colorectal carcinoma (HNPCC) as defined by the Collaborative Group in HNPCC. (Hakala *et al.*, 1991). There is current debate as to whether this syndrome is associated with a significant increase in breast cancer risk (Lynch *et al.*, 1991).

The recombination-exclusion map presented in Figure 5 reveals that 5 linked pedigrees contain critical recombinants in affected individuals involving markers on 17q. Two critical recombinants exclude regions telomeric to 42D6 and 3 exclude regions centromeric to MFD188. 42D6 and MFD188 flank a region 5 to 10 cM in length. Pedigree structures with markers illustrated in Figures 6a and 6b reveal that, although the precise point of recombination is difficult to identify because of the extensive nature of some pedigrees, the evidence suggests that a breast cancer susceptibility gene might be located between these markers.

Because all bilateral breast and ovarian cancer alleles typed at 42D6 and MFD188 also co-segregated with other breast cancer or other ovarian cancer in the same pedigree, it seemed reasonable to assume that most cases of ovarian cancer in Edinburgh pedigrees were linked to a 17q gene. Linkage analysis in those pedigrees with either bilateral breast cancer or ovarian cancer was therefore employed under new assumptions that these phenotypes endowed the individual with a likelihood of carrying the gene equivalent to that for breast cancer at age 25. This resulted in overall increases in summated LOD scores (Table X).

The association between breast cancer and ovarian cancer is well known (Lynch and Kullander, 1987b) and two recent studies have reported a high proportion of allele loss using CMM86 in ovarian tumours (Sato *et al.*, 1991; Foulkes *et al.*, 1991). Bilateral breast cancer has also been associated with familial disease (Iselius *et al.*, 1991). According to the retinoblastoma model (Knudson, 1985) the presence of a breast cancer gene would result in a calculable risk of one or subsequent tumours in either breast. The actual number of tumours per gene carrier would be expected to fit closely to a Poisson distribution. Thus the probability of a second event would be less than that of a first event, and further reduced by mastectomy. A third event is rendered impossible after bilateral (complete) mastectomy.

A mechanism to explain why both ovarian and breast cancer might be precipitated by the same genetic lesion could centre either on the cyclical nature of epithelial proliferation in these organs, or on their sensitivity to steroid hormones. The response of breast cancer and ovarian cancer to hormonal stimuli is complex, but it is at least possible that the proliferative effect of steroids may promote a second-copy mutation of a breast or ovarian epithelial cell compromised by an inherited first-copy defect (Lemon, 1991).

Interestingly, there were no cases of endometrial cancer in the line of descent of BRCA I carriers in Edinburgh pedigrees. The endometrium, like the breast and the ovary, responds to steroidal hormones in a cyclical manner, and endometrial carcinoma has an age-incidence

curve similar to those for other gynaecological malignancies. Some epidemiological studies also associate it with familial breast cancer, although not as part of HNPCC (Lynch *et al.*, 1991).

Cancer of the colon and breast malignancy are also associated in the syndrome HNPCC. In Edinburgh pedigrees, alleles at 42D6 and MFD188 in 9 out of 13 individuals with colon cancer segregated elsewhere in the pedigree with breast cancer. Since it has been claimed that as many as 80% of colon cancers may develop as a result of genetic predisposition (Cannon-Albright *et al.*, 1988) and that 1 in 23 Scottish males and 1 in 33 females develop colon cancer during their lifetime, then the results from Edinburgh pedigrees carrying BRCA I mutations suggest this gene might also predispose to colon cancer.

In other types of cancer, the proportion of individuals with alleles co-segregating with breast cancer was 31 out of 44. There were insufficient numbers to draw conclusions about linkage of specific cancer types to BRCA I.

One case of male breast cancer occured in the 15 Edinburgh pedigrees subjected to linkage analysis. This individual in pedigree 11 appeared to be a carrier of BRCA I gene mutation (Figure 6b). Although germline mutations of the androgen receptor gene have been reported in two brothers with male breast cancer (Wooster *et al.*, 1992), no previous evidence for linkage of male and female breast cancer to specific loci exists in the literature.

The posterior probability of an individual in a breast cancer family developing breast cancer is a function of the mode of genetic transmission, age-specific incidence of sporadic disease, prior probability of a breast cancer gene in the sibship, age-specific penetrance of the gene and age of the individual for whom risk is being assessed. Linkage analysis provides additional data for determining posterior probability and relies upon an accurate estimation of recombination distances between flanking markers and the gene. Computer programs which assess genetic risk and take into account linked marker data include modifications of LIPED (Ott, 1976) by Winter (1985). Estimates of probability of inheritance of BRCA I mutation in linked pedigrees can be made without computerised aids. In individuals from linked Edinburgh pedigrees, calculations of probability of inheritance of BRCA I mutation ranged from 0.8% to 98.9%. Lifetime risk of breast cancer ranged from 9.8% to 87.2%. Although real ethical problems must be considered, these figures can be useful in a clinical setting through identification of women most likely to benefit from organ screening or prophylactic measures.

Individuals belonging to families in which multiple cases of breast cancer and/or ovarian cancer occur in a line of descent consistent with autosomal dominant genetic transmission will have a high prior probability of genetic inheritance of a breast cancer gene. Where there is also clear evidence for linkage of the disease to 17q markers within the same pedigree, then unaffected women with marker alleles co-segregating with the disease will be at much higher risk than is

currently estimated in analyses devoid of linkage data. Conversely, unaffected women with flanking marker alleles which do not co-segregate with the disease elsewhere in the pedigree will be at lower risk than current estimates. These risks will be more accurately assessed in large linked pedigrees with multiple cases of breast cancer and in which LOD scores are greater than +1.5.

Although immediate clinical application of linkage analysis data is limited, the definitive identification BRCA I is anticipated. Only then will it be possible to screen individuals for relevant mutations without reference to laborious family studies in order to define carrier status. Instead, a single blood sample should suffice to assign a high or low level of disease risk to breast cancer family members.

**ii) Penetrance, age-incidence and survival in 17q breast cancer gene carriers.**

Genetic penetrance of the BRCA I gene recorded here (Figure 10) is similar to that observed by the International Breast Cancer Linkage Consortium (Easton *et al.*, 1993). The plateau of the curve appears to be reached at earlier age than that estimated in segregation analysis studies (Claus *et al.*, 1991; Iselius *et al.*, 1991). One explanation for this could be that BRCA I-linked pedigrees are chosen precisely because they contain multiple cases of breast cancer and so are pre-selected for high rates of disease penetrance. Equally there may be genetic heterogeneity within the totality of heritable breast cancer, with approximately equal contribution from a highly penetrant BRCA

I gene and much less penetrant additional genes (such as the p53 and the ataxia telangiectasia genes) and perhaps other genes which remain to be identified. Although germline p53 gene mutation is considered highly penetrant in the sarcomas, carcinomas and leukaemias of classic Li-Fraumeni syndrome families, recent studies suggest it may be much less so in heritable breast cancer (Prosser *et al.*, 1992). Furthermore, if heterozygosity for the ataxia telangiectasia gene does indeed signify increased risk of breast cancer, then it appears likely that its penetrance is less than that of BRCA I. (Swift *et al.*, 1990; Borresen *et al.*, 1990).

The genetic model produced by segregation analysis (in which early onset of disease is assumed to be relatively more likely in gene carriers than non-gene carriers and in which modifications of the analysis allow bilateral disease or diagnosis of ovarian cancer to increase the likelihood of carrier status) affects overall odds of linkage. It was therefore expected that the age-incidence curve for BRCA I mutation carriers would be shifted to the left of that for the general population (Figure 11) and that bilateral breast cancer should occur in 13 out of 35 (37%) of affected BRCA I mutated individuals. This figure is similar to the 33% bilaterality rate computed for "totality of breast cancer" gene carriers in segregation analysis studies (Iselius *et al.*, 1991).

It is a widely held axiom that early-onset breast cancer is a more aggressive disease than that which occurs in older women (Nemoto *et al.*, 1980). At least one study, however, reveals that although heritable

breast cancer has an age-incidence curve shifted in favour of early-onset disease, the familial form of malignancy may result in significantly better survival than that in breast cancer as a whole (Albano *et al.*, 1982).

The survival curve for the affected BRCA I mutation carriers (Figure 12) may indicate a more optimistic prognosis than that for breast cancer in the general population. Is it possible that BRCA I mutation carriers have a spurious survival advantage based on favourable tumour stage or selection for longevity? There is no evidence from epidemiological studies that tumour stage at diagnosis in breast cancer pedigrees differs from that in the general population (Albano *et al.*, 1982), and in Edinburgh families no selective screening of individuals had been instituted prior to BRCA I genotyping. In the past, DNA analysis through blood sampling was only possible in surviving individuals, thus selection of pedigrees with a bias towards prolonged breast cancer survival was likely in order to obtain linkage data. The current procedure for genotyping at loci 42D6 and MFD188 however involves DNA typing from additional deceased individuals since fixed paraffin-wax embedded tissue can be utilised which eliminates the bias towards breast cancer survival. This absence of bias is confirmed by the lack of any correlation between breast cancer survival in linked pedigrees and maximal odds of linkage for each family (Table XIII).

It is therefore possible that the survival data reflects a real phenotypic effect of BRCA I mutation. If these findings are confirmed in other larger series, than future analysis of survival data from BRCA I

mutation carriers in breast screening programmes must take into account this pre-existing survival advantage to avoid an over-optimistic interpretation of mortality reduction in the high-risk screened cohort.

**Table XIII**

Maximal odds of linkage, mean survival of deceased and surviving individuals with breast cancer and ranking of these parameters in 8 BRCA1-linked pedigrees.

## Table XIII

| Pedigree Identification number | Maximal Odds of Linkage (%) | Rank | Mean Survival of Deceased Breast Cancer Cases /Years | Rank | Mean Survival of Surviving Breast Cancer Cases /Years | Rank |
|---|---|---|---|---|---|---|
| 2 | 99.9 | 1 | 1.5 | 8 | 18.3 | 2 |
| 11 | 99.2 | 2 | 5.5 | 6 | 4.0 | 7 |
| 30 | 95.9 | 3 | 11.0 | 4 | 2.5 | 8 |
| 2000 | 94.6 | 4 | All Alive | 1= | 7.5 | 5 |
| 37 | 92.3 | 5 | 3.5 | 7 | 29.5 | 1 |
| 1 | 88.8 | 6 | 6.0 | 5 | 17.0 | 3 |
| 16 | 85.5 | 7 | 13.0 | 3 | 5.0 | 6 |
| 3 | 79.2 | 8 | All Alive | 1= | 10.7 | 4 |

## 3. Structural chromosome analysis.

The complex chromosomal variants found in the pedigree illustrated in Figure 13 suggest that there may be some heritable predisposition to cytogenetic aberrations in this family. A putative causative gene might be inherited in autosomal dominant fashion with consequent accumulation of non-lethal chromosomal variants in both gene carriers and non-gene carriers alike. In this situation, de-novo chromosome rearrangements will occur only in gene carriers at meiosis. Non-gene carriers will inherit previously accumulated anomalies and pass on these to their progeny. Perhaps a gene predisposing to cytogenetic aberrations also produces a generalised genetic instability in which breast and other cancers become more likely. In these respects it appears to share striking characteristics with the ataxia telangiectasia gene (Gatti *et al.*, 1988).

Chromosome analysis in the non Li-Fraumeni syndrome cancer family illustrated in Figure 14 reveals two individuals with chromosome abnormalities. No individuals exhibited detectable abnormal phenotypes. One individual has Turner's syndrome (XO), the other an inversion involving chromosome 5. Both individuals inherited a germline p53 gene mutation. It is recognised that cytogenetic analyses of peripheral blood leukocytes in Li-Fraumeni syndrome family members reveal chromosome loss in a greater than expected proportion of cells (Bischoff *et al.*, 1990) and it is therefore possible that the constitutional chromosomal anomalies found in two carriers of the mutant p53 gene reflect a general chromosome instability

produced by mutant p53 protein; an instability which is apparently not exhibited in carriers of a 17q breast cancer susceptibility cancer gene.

Table XIIb reveals that there is a significant reduction in single cell chromosome anomalies in general (and in chromosome loss and fragile sites expression specifically) in age-matched BRCA I mutation carriers unaffected by breast cancer compared with their non-gene carrying relatives. Figure 15 shows that no genomic regions are preferentially targeted for abnormalities which occur in BRCA I carriers. This analysis provides no support for a hypothesis involving increased DNA instability as a mechanism of action for BRCA I; in fact the opposite appears more likely. Interestingly, a comparable observation was reported by Mitchell et al., (1991). They found that lymphocytes in women unaffected by breast cancer but with a strong family history of the disease exhibited no increases in susceptibility to fragile site expression and induction by aphidicolin compared with controls. Perhaps carriers of breast cancer susceptibility gene mutation, heterozygous at the BRCA I locus, compensate in some way for the reduced level of crucial BRCA I gene product though increased expression of other genes important in cell cycle control and which act secondarily to stabilise cell division, thereby increasing rates of DNA repair prior to mitosis.

## Conclusion

These genetic studies on peripheral blood lymphocytes of individuals from breast cancer pedigrees have assisted in the localisation of a major breast cancer susceptibility gene (BRCA I) on chromosome 17q 12-21 through linkage analysis. They have enabled high-risk women from both BRCA I and p53 gene mutation carrying families to be identified and discriminated from their low-risk, non-gene carrying relatives. Structural chromosome analysis has suggested a role for DNA instability as a mediator of cancer risk in p53 but not BRCA I mutation pedigrees. Preliminary survival data may indicate an improved prognosis in BRCA I mutation carriers with breast cancer compared with survival in breast cancer in general.

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